Microbial content and anti-microbial activity of Namibian traditionally fermented milk

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DECLARATION

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ABSTRACT

The incorporation of bacteriocins as biopreservatives into model food systems has been studied extensively and has been shown to be effective in the control of pathogenic and spoilage microorganisms. However, a more practical and economic option of incorporating bacteriocins into foods can be by direct addition of bacteriocin-producing cultures into food. In this study five samples of traditionally fermented Omaere was sourced from households in Namibia. The microbial consortium present was isolated and enumerated on six different selective media that included deMan, Rogosa and Sharpe Medium (MRS) supplemented with cycloheximide for lactobacilli (MRS+C), MRS supplemented with vancomycin for leuconostocs (MRS+V), MRS supplemented with ethanol for acetic acid bacteria, M17 agar for lactococci, and Chloramphenicol Glucose Agar (CGA) and Potato Dextrose Agar (PDA) for yeasts. The highest enumeration values obtained for Omaere samples 2 and 3 were from MRS+V used for the growth of *Leuconostoc* spp. However, for samples 1, 4 and 5 the highest values were obtained from MRS+C used for the growth of lactobacilli. This variance among samples can be attributed to the inconsistency in the preparation methods of traditionally fermented milks.

After isolation and enumeration of the microbes present in each milk sample, the Harrison Disc method was used to select bacteria and yeast colonies for further testing. The primers 27F and R1492 were used to amplify a 1.5 kilobase (kb) fragment of the 16S ribosomal RNA (rRNA) gene of the selected bacteria colonies using the polymerase chain reaction (PCR). The primers ITS4 and ITS5 were used to amplify a 600 base pair fragment of the internal transcribed spacer (ITS) regions of the fungal rRNA gene and NL1 and NL4 was used to amplify the D1/D2 domain of the 26S rRNA gene of the selected yeast isolates. The resulting PCR products were sequenced and compared to sequences listed in NCBI database using the BLAST algorithm and identified according to the closest relative. The LAB found in Namibian fermented milk Omaere belonged to the genus *Lactobacillus*, with the predominant species *Lactobacillus plantarum* (52%) and in lesser numbers *Lactobacillus paracasei* subsp. *paracasei* (12%), *Lactobacillus paraplantarum* (8%), *Lactobacillus kefiri* (8%), and *Lactobacillus casei* (2%). The yeasts isolated were *Kazachstonia unispora* formerly known as *Sacchromyces unisporus (9%), Saccharomyces cerevisiae (8%)* and *Candida pararugosa (2%).*

Pure cultures of the *Lactobacillus* spp. isolated were used to ferment milk that was inoculated with *Listeria monocytogenes* and *Escherichia coli* and their interaction was monitored over time. After 48 h of fermentation, *L. monocytogenes* was not detected in milk samples

inoculated with *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus casei* subsp*. paracasei.* In contrast, when *Lactobacillus kefiri* was inoculated with the two foodborne pathogens, after 48 h of fermentation the concentration of *L. monocytogenes* was reduced by 4 log and was not detected after 72 h. In milk fermented without the addition of starters, the concentration of *L. monocytogenes* was only reduced by 2.8 log after 72 h of fermentation. In the milk with *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus casei* subsp. *paracasei* after 48 h of fermentation the *E. coli* concentration was reduced by 4 log and after 72 h of fermentation no *E. coli* was detected. In contrast, fermentation with *Lactobacillus kefiri* after 48 h resulted in a decreased concentration of 1 log and at the end of the 72 h the *E. coli* concentration was only reduced by 1.7 log. In milk fermented without the addition of starter the concentration of *E. coli* was only reduced by 1.6 log after 72 h of fermentation.

The results obtained in this study show that three of the four LAB strains isolated from the Namibian fermented milk, Omaere namely, *L. plantarum, L. paraplantarum, L. parcasaei* subsp. *paracasei* had inhibitory effect against the studied foodborne pathogens. Therefore, after further characterization of the types of antibacterial agents that are produced by these LAB, they could be considered as potential candidates for development of starter cultures that can be used for the production of microbiologically safe commercial fermented milk products. However, the *L. kefiri* strain used in this study is likely not to be used as starter culture as it took longer to eliminate or failed to eliminate the foodborne pathogens used in the study.

UITTREKSEL

Die gebruik van bakteriosiene as bio-preserveermiddels in voedselsisteme is omvattend bestudeer en is bewys om doeltreffend te wees in die beheer van patogene en mikroörganismes. Daar kan egter 'n meer praktiese en ekonomiese opsie wees vir die integrasie van bakteriosiene in voedsel deur direkte toevoeging van bakteriosien-vervaardigende suurselkulture tot kosse. Hierdie studie beskryf vyf monsters van tradisioneel gefermenteerde Omaere, afkomstig van huishoudings in Namibië. Die mikrobiese konsortium teenwoordig is geïsoleer op ses verskillende selektiewe media, insluitend deMan, Rogosa and Sharpe Medium (MRS) aangevul met cycloheximied vir lactobacilli, MRS aangevul met vancomycin vir leuconostocs (MRS+V), MRS aangevul met etanol vir asynsuur bakterieë, M17 agar vir lactococci, asook Chlooramfenikol Glukose Agar (CGA) en aartappel Dekstrose Agar (PDA) vir gis. Die hoogste tellings verkry vir Omaere monsters 2 en 3, was met MRS+V wat gebruik word vir die groei van *Leuconostoc* spp. Vir die monsters 1, 4 en 5 is die hoogste waardes verkry op MRS+C wat gebruik word vir die groei van lactobacilli. Hierdie verskille tussen die monsters kan toegeskryf word aan die verskille in voorbereidingsmetodes van tradisionele suurmelkdranke.

Na die isolasie en tel van die mikrobes in elke melkmonster, is die Harrison Skyfmetode gebruik om bakterieë en gis kolonies vir verdere toetse te kies. Die priemstukke 27f en R1492 is gebruik om 'n 1.5 kilobasis (kb) fragment van die 16S ribosomale RNA (rRNA) geen te amplifiseer van die gekose bakteriële kolonies met behulp van die polymerase-kettingreaksie (PKR). Die priemstukke ITS4 en ITS5 is gebruik om 'n 600 basispaar fragment van die interne getranskribeerde spasie van fungi ribosomale DNA (rDNA) te amplifiseer en NL1 en NL4 is gebruik om die D1 en D2 area te amplifiseer. Die gevolglike PKR produkte se basispaaropeenvolging is bepaal en in die NCBI databasis met behulp van die BLAST algoritme geïdentifiseer volgens die naaste familielid. Die melksuurbakterieë in Namibiese gefermenteerde melk, Omaere behoort aan die genus *Lactobacillus*, met die oorheersende spesie *Lactobacillus plantarum* (52%) en in mindere getalle *Lactobacillus paracasei* subsp. *paracasei* (12%), *Lactobacillus paraplantarum* (8%), *Lactobacillus kefiri* (8%), en *Lactobacillus casei* (2%). Die gis geïsoleer was *Kazachstonia unispora* voorheen bekend as *Sacchromyces unisporus* (9%), *Saccharomyces cerevisiae* (8%) en *Candida pararugosa* (2%).

Suiwer kulture van die *Lactobacillus* spp. is gebruik om melk wat ingeënt is met *Listeria monocytogenes* en *Escherichia coli* te fermenteer. Hul interaksie is gemonitor met verloop van tyd en na 48 uur van fermentasie, is *L. monocytogenes* nie bespeur in melkmonsters wat

gefermenteer is met *Lactobacillus plantarum*, *Lactobacillus paraplantarum* en *Lactobacillus casei* subsp. *paracasei* nie. In teenstelling, wanneer *Lactobacillus kefiri* ingeënt is saam met die twee voedselverwante patogene is die konsentrasie van die *L. monocytogenes* verminder met 4 log na 48 uur en die patogeen is nie opgespoor na 72 uur nie. In melk gefermenteer sonder die byvoeging van kulture, is die konsentrasie van *L. monocytogenes* net verminder met 2,8 log na 72 h van fermentasie. In die melk met *Lactobacillus plantarum*, *Lactobacillus paraplantarum* en *Lactobacillus casei* subsp. *paracasei* na 48 h van fermentasie, is die *E. coli* konsentrasie verminder met 4 log en na 72 h is geen *E. coli* bespeur nie. In teenstelling, fermentasie met *Lactobacillus kefiri* het na 48 h gelei tot 'n afname in die konsentrasie van 1 log en aan die einde van die 72 uur is die *E. coli* konsentrasie verminder met 1,7 log. In melk gefermenteer sonder die byvoeging van die suurselkulture, is die konsentrasie van *E. coli* slegs verminder met 1,6 log na 72 h van fermentasie.

Die resultate wat verkry is in hierdie studie toon dat drie van die vier melksuurbakterieë-stamme wat geïsoleer is uit die Namibiese gefermenteeerde melk, Omaere, naamlik *L. plantarum, L. paraplantarum, L. parcasaei* subsp. *paracasei,* ‘n inhiberende effek teen die spesifieke voedselverwante patogene gehad het. Dus, na verdere karakterisering van die tipe antibakteriese middels wat geproduseer word deur hierdie melksuurbakterieë kan hulle oorweeg word as potensiële kandidate vir die ontwikkeling van suurselkulture wat gebruik kan word vir die produksie van mikrobiologiese veilige, kommersieël gefermenteerde melkprodukte. Maar die *L. kefiri* stam wat in hierdie studie geisoleer is, kan waarskynlik nie gebruik word as 'n suurselkultuur nie, aangesien dit langer neem om die voedselverwante patogene uit te skakel.

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Chapter 1

Introduction

Milk and products derived from the milk of dairy cows can harbor a variety of microorganisms and can be important sources of foodborne pathogens (Varnam & Sutherland, 2001). The presence of foodborne pathogens in milk may be due to direct contact with contaminated sources in the dairy farm environment and due to excretion from the udder of an infected animal. *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium bovis* and *Staphylococcus aureus* are examples of common foodborne pathogens found in milk (Harding, 1999; Krause & Hendrick, 2011).

Milk has been preserved since early times by fermentation involving the microorganisms present in the raw milk (Gould, 1999; Tamine, 2006). The fermented milks differ from region to region depending on the indigenous microbes present, environmental conditions such as temperature, origin and quality of the milk, processing and sanitary conditions (Kurmann, 1994; Tamine, 2006). In most cases, the same vessels are constantly used to ferment milk and fresh milk is usually added to on-going fermentation mixtures. Fermented food products are often considered to be microbiologically safe because of the low pH and production of antimicrobial substances by fermenting organisms (Tamine & Robinson, 1988; Kurmann, 1994; Hutkins, 2008).

A wide variety of microbes can be responsible for the fermentation of milk, including lactic acid bacteria (LAB), acetic acid bacteria (AAB), yeasts and mycelial fungi (Hutkins, 2006). LAB are generally recognised as safe (GRAS) and play a key role in food fermentations where they not only contribute to the development of the desired sensory properties in the final product but also due to their anti-microbial activity (Wood & Holzapfel, 1995; Widyastuti & Febrisiantosa, 2014). These bacteria are Gram-positive, non-motile, non-spore forming, non-respiring and are characterised based on the rod or coccus shape of the cells and their negative catalase activity. LAB ferment carbohydrates and produce lactic acid as the major end-product. LAB are classified into two major groups based on the metabolic pathways employed in the fermentation of carbohydrates, namely homo-fermentative and hetero-fermentative (Benniga, 1990; Wood & Holzapfel, 1995; Caplice & Fitzgerald, 1999). The inhibitory action of LAB has been attributed to the accumulation of primary metabolites, such as lactic and acetic acids, ethanol and carbon dioxide, as well as to the production of other antimicrobial compounds, such as formic and

benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins (Maragkoudakis *et al*., 2006; Ljungh & Wadstrom, 2009).

The presence of yeasts in dairy products lead to an end-product which differs in physico-chemical properties from those made with pure LAB starters. Yeasts promote symbiosis among microorganisms present and forms carbon dioxide adding fizziness and a specific aroma and slight yeasty taste (Caplice & Fitzgerald, 1999; Boulton & Quain, 2001). By incorporating LAB which produces antimicrobial compounds into commercial starter cultures, the use of chemical preservatives such as sodium benzoate and sodium metabisulphite can be reduced. The aim of this study is to isolate, enumerate and identify LAB and yeasts from Namibian traditionally fermented milk Omaere and to test the identified LAB for antimicrobial activity against *L. monocytogenes* and *E . coli*.

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Chapter 2

Literature Review

A. Background

Milk is one of the most important mammalian foods and is rich in the protein casein, which gives it the characteristic white colour (Harding, 1995). The most abundant carbohydrate present in milk is the disaccharide lactose, commonly known as milk sugar (Jensen, 1995). In the course of human evolution it was recognised that the milk of other mammals was equally satisfying in meeting physiological demands for moisture, energy, and nutrients. Milk from domesticated mammals such as cows, buffalo, sheep, goats, horses, camels and yak is mostly used for human consumption in different parts of the world (Varnam & Sutherland, 2001; Robinson, 2002). Early in history the ability to digest milk was limited to children as adults did not produce lactase, an enzyme necessary for digesting lactose. During fermentation lactic acid bacteria convert 25 - 50% of lactose to lactic acid consequently reducing the amount of lactose in fermented milk compared to raw milk (Chandan *et al*., 2008). This reduced content of lactose in fermented milk is an important factor for better tolerance of fermented milks by lactose intolerant individuals (Tamang, 2015).

Milk fermentation is the most widely used method for milk preservation. Historically the fermentation of milk can be traced back to around 10 000 to 15 000 years ago, coinciding with the shift from hunters and food gatherers to food producers. It is likely that fermentation arose spontaneously from indigenous populations of microorganisms found in milk and the environment (Kurmann, 1994; Robinson, 2002; Tamine, 2006). Initially, the objective of fermenting milk was to produce lactic acid to extend the shelf life and storage of milk in the absence of refrigeration. However, other benefits are derived from the consumption of fermented milk such as reducing risks of heart attacks in hypocholesterolemic individuals by lowering their plasma cholesterol (Mann & Spoery, 1974; Tamang, 2015) and the lactic acid bacteria from fermented milk have potential anticarcogenic activity (Tamang, 2015). Furthermore, fermented milk provides income to the rural poor, especially to women and children and, therefore, serves as a means of employment in rural areas (Bille, 2007). Despite the benefits of fermented milk products, traditional fermentation is uncontrolled and can possibly be exposed to contamination with foodborne pathogens.

B. Milk as a source of pathogens

In addition to being a nutritious food for humans, milk provides a favourable environment for the growth of microorganisms. Milk is synthesized by cells within the mammary gland and is sterile when secreted into the alveoli of the udder. Beyond this stage of milk production, bacterial contamination can occur from three main sources, namely within the udder, outside the udder, and from the surface of equipment used for milk handling and storage (Varnam & Sutherland, 2001; Jay *et al*., 2005). Bacterial contamination from within the udder is frequently a result of mastitis, an inflammatory disease of the mammary tissue. A cow with mastitis has the potential to shed large numbers of microorganisms into her milk. The influence of mastitis on the total bacterial count of bulk milk depends on the type of bacteria, the stage of infection and the percentage of the herd infected. The exterior of the cow's udder and teats can contribute to microorganisms that are naturally associated with the skin of the animal, as well as microorganisms that are derived from the environment in which the cow is housed and milked. The influence of infected cows on total bacterial counts depends on the extent of soiling of the teat surface and the udder preparation procedures employed. However, the degree of cleanliness of the milking system influences the total bulk milk bacterial count more than any other factor (Varnam & Sutherland, 2001; Jay *et al*., 2005). Milk residue left on equipment contact surfaces supports the growth of a variety of microorganisms. Cleaning and sanitizing procedures can influence the degree and type of bacterial growth on milk contact surfaces by leaving behind milk residues that support growth, as well as by creating conditions that might select for specific microbial groups. Even though equipment surfaces may be considered efficiently cleaned with hot water, heat resistant bacteria (thermoduric) may endure in low numbers. If milk residue is left behind (milk stone), growth of these types of organisms, although slow, may persist (Varnam & Sutherland, 2001; Jay *et al*., 2005).

Foodborne pathogens often do not change the odour, taste or appearance of food and it may not be easy to assess the microbial safety of a product without performing multiple microbiological tests (Jay *et al*., 2005). *Bacillus cereus, Escherichia coli, Listeria monocytogenes*, *Mycobacterium bovis* and *Staphylococcus aureus* are examples of common foodborne pathogens isolated from milk (Jay *et al*., 2005).

Escherichia coli

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium (Donnenberg, 2002). Most strains of *E. coli* do not cause illness and live in the intestinal tracts of healthy humans and animals (Donnenberg, 2002), however, some strains such as *E. coli* O157:H7 are pathogenic. Symptoms of illness include bloody diarrhoea and abdominal cramps. In some cases, particularly in young children, *E. coli* O157:H7 infection causes haemolytic uremic syndrome, during which red blood cells are destroyed and kidneys are damaged (Varnam & Sutherland, 2001; Britz & Robinson, 2008). *Escherichia coli* is transmitted to humans primarily through the consumption of contaminated foods, such as raw or undercooked ground meat products, unpasteurised milk and contaminated raw vegetables. Its significance as a public health problem was recognised in 1982, following an outbreak in the United States of America (Manning, 2010).

Escherichia coli has been isolated from fermented foods indicating that these bacteria are capable of growing in the food or surviving the fermentation process. Cereal and milk-based fermented products used as weaning foods are a major source of *E. coli*. Nyatoti *et al.* (1994) reported that out of 12 samples of naturally fermented milk used as weaning foods, 2 were contaminated with *E. coli.* In South Africa, Kunene *et al*. (1999) reported that 7% of the fermented sorghum meal samples contained *E. coli and* 20% of *Enterobacteriaceae* from fermented milk was also identified as *E. coli*. The presence of *E. coli* in any food product is a possible indication of fecal contamination and according to the Department of Agriculture, Forestry and Fisheries of South Africa, *E. coli* should be absent in 1 ml of milk.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, rod-shaped bacterium. It is the causative agent of listeriosis, a serious infection caused by ingesting the bacteria through contaminated food (Liu, 2008). There are two types of listeriosis, namely a self-limiting gastrointestinal illness and invasive listeriosis which can be life threatening. The gastrointestinal form is characterised by flu-like symptoms (diarrhoea, vomiting and fever) that may occur after ingestion of contaminated food. However, invasive listeriosis may have an onset time of two to six weeks and adults may experience septicaemia, meningitis and endocarditis, whereas unborn fetuses may develop abscesses in their liver, lungs and other organs often resulting in spontaneous abortion and still

birth. Surviving children may be seriously ill with meningitis and neurological impairment (Goldfine & Shen, 2007; Ryser & Marth, 2007).

Listeria monocytogenes is commonly found in soil and water. Animals can carry the bacteria without appearing ill and can contaminate foods of animal origin, such as meats and dairy products. These bacteria have also been found in a variety of foods, including uncooked meats and vegetables, unpasteurised (raw) milk and cheeses, and cooked or processed foods, including processed (or ready-to-eat) meats, and smoked seafood. Pasteurization of milk effectively destroys *L. monocytogenes*, however, post-pasteurization contamination can occur within the processing plant. *Listeria monocytogenes* is capable of growing at refrigeration temperatures, therefore, even very low numbers in processed dairy products can multiply to dangerous levels despite proper refrigeration. An adequate sanitation program and good hygiene practices are essential in food processing and handling areas to avoid *L. monocytogenes* contamination (Goldfine & Shen, 2007; Liu, 2008).

The first reports of the presence of *Listeria* in food are associated with dairy products, and soft cheeses and non-pasteurised milk are the most common sources of these bacteria (Ryser & Marth, 2007). The consumption of milk and dairy products contaminated with *L. monocytogenes* can lead to cases of listeriosis or the outbreak of this disease. Two large outbreaks in human populations were associated with the consumption of soft cheeses. In California, from June to August 1985, 142 people became ill of whom 48 died (Linnan *et al*.,1988), and in Switzerland, in the period from 1983 to 1987, 122 cases were recorded, of which 34 individuals died (Bell & Kyriakides, 1998).

Bacillus cereus

Bacillus cereus is a Gram-positive, aerobic, spore-forming rod normally present in soil, dust and water. It is a common contaminant in many food types, including milk, and is a significant cause of foodborne illness worldwide. *Bacillus cereus* produces two toxins that can cause diarrhoea and vomiting (Bottone, 2010). The symptoms are generally mild and transient, lasting no longer than 24 h. Their spores survive pasteurization and psychotropic strains of *B. cereus* limit the keeping quality of milk stored at temperatures higher than 6°C. The highest numbers of *B. cereus* spores in raw milk are found during the grazing season, mainly due to contamination of the teats by soil (Varnam & Sutherland, 2001; Adams & Moss, 2008; Logan & De Vos, 2011).

Mycobacterium bovis

Mycobacterium bovis is a slow-growing, aerobic bacterium and the causative agent of tuberculosis in cattle known as bovine tuberculosis (BTB). This is a disease characterised by progressive development of specific granulomatous lesions or tubercles in lung tissue, lymph nodes or other organs (Ayele *et al*., 2004; Thoen *et al*., 2006). BTB is a zoonotic disease and humans are generally infected by eating or drinking contaminated unpasteurised milk products from *M. bovis* infected cattle. *Mycobacterium bovis* can also be spread through the air when an infected person coughs or sneezes. However, airborne transmission is less common than transmission through the consumption of food products (Thoen *et al*., 2006). In Africa, BTB represents a potential health hazard to both animals and humans, as nearly 85% of cattle and 82% of the human population live in areas where the disease is prevalent or only partially controlled (Cosivi *et al.*, 1998).

Staphylococcus aureus

Staphylococci are facultative Gram-positive cocci and occur in microscopic clusters resembling grapes (Honeyman *et al*., 2001). The presence of *S. aureus* in raw milk is generally caused by cows with mastitis, handlers or deficient hygiene. The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows. *Staphylococcus aureus* produces toxins that destroy cell membranes and can directly damage milk-producing tissue (Honeyman *et al*., 2001). Mastitis infections are spread from infected cows to non-infected cows during milking via milking machines, milkers' hands, contact with milk secretions in stalls and flies can serve as vectors of *S. aureus*, transferring it from one animal to another (Freeman-Cook & Freeman-Cook, 2006).

C. Milk fermentation

Milk has been preserved since early times by lactic acid fermentation. Lactic acid fermentations can be divided into two broad categories distinguishable by the end products of glucose hydrolysis, namely homo-fermentation and hetero-fermentation (Adams & Moss, 2008). Homo-fermenters such as *Lactococcus* spp., *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus* and *Streptococcus thermophilus,* convert glucose to 6-phosphogluconate using the Embden-Meyerhof (EM) pathway (Adams & Moss, 2008). The end product in this fermentation pathway is the production of more than 90% lactic acid, which is responsible for the refreshing

taste, preservation of fermented milk products and gel formation (Adams & Moss, 2008). Hetero-fermentative LAB, such as *Leuconostoc lactis* and *Leuconostoc mesenteroides* subsp*. cremoris* and *Lactobacillus fermentum,* lack aldolases and, therefore, cannot ferment sugar via the glycolytic pathway (Adams & Moss, 2008). The pentose phosphate pathway is used instead of the EM pathway of glycolysis. This type of fermentation produces ethanol and carbon dioxide in addition to lactic acid (Benniga, 1990; Adams & Moss, 2008).

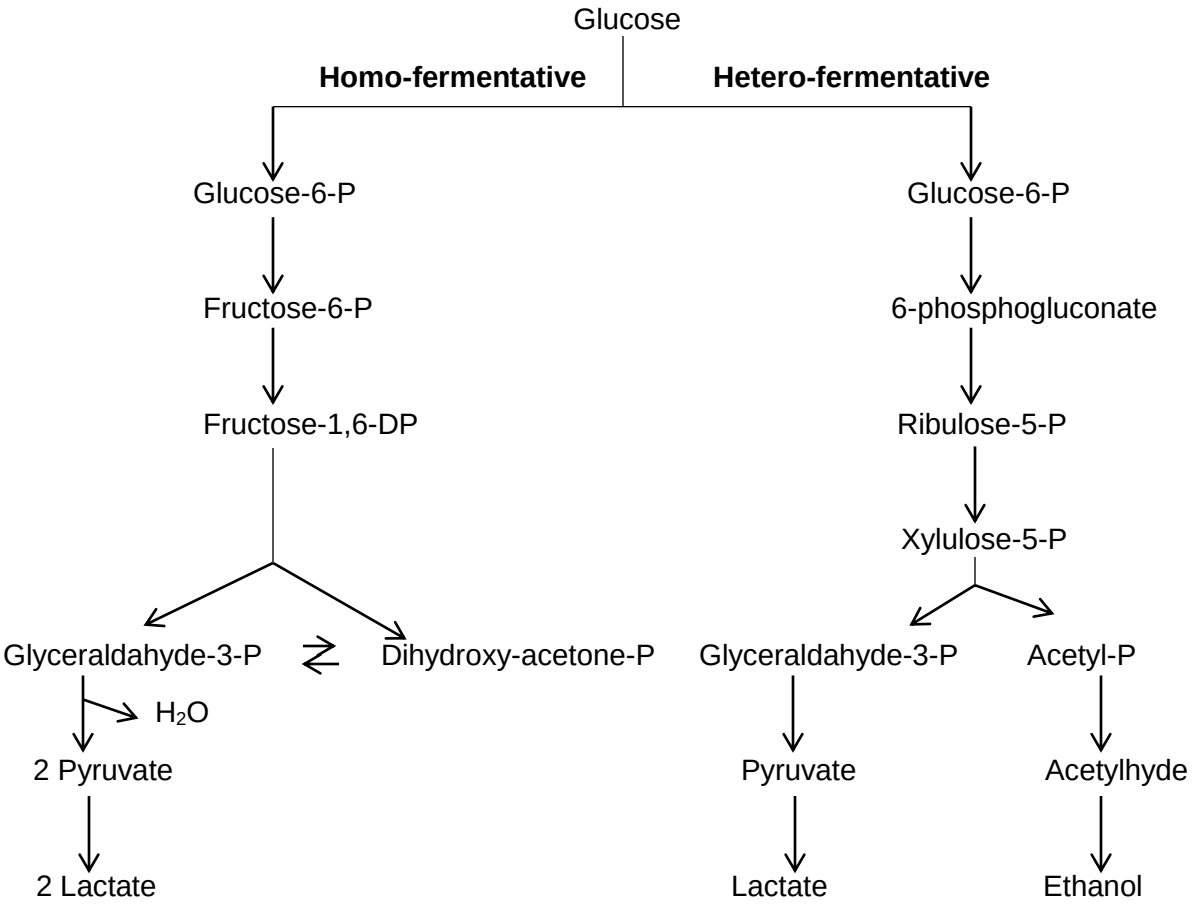

Figure 1 The pathway for glucose dissimilation by homo-fermenters and hetero-fermenters (Adams & Moss, 2008).

Traditionally, milk fermentation was initiated by the natural microorganisms in the milk, originating from the environment, processing equipment, processors or by the back sloping technique, adding small amounts of previously fermented milk as a starter into fresh milk (Abdelgadir, 1998; Savandago *et al*., 2004 Mufandaedza *et al.*, 2006). Due to the spontaneous nature of the fermentation, this traditional method results in a product with varying taste and

flavour often of poor hygienic quality depending on the predominant microorganism present in the milk (Mufandaedza *et al.*, 2006). Foodborne pathogens are often isolated from traditionally fermented milk (Beukes *et al*., 2001; Savandago *et al*., 2004; Akabanda *et al*., 2010; Schutte, 2013). This is of concern because these foods are also used as weaning foods and according to Nout *et al*. (1989), mortality and morbidity rates due to diarrheal diseases are highest in infants during the weaning period. The safety of fermented milk products, therefore, becomes a major public health concern. Greater control of milk fermentation is achieved when microorganisms isolated from traditionally fermented milk is deliberately added as starter cultures to pasteurised milk on industrial scale. This industrially produced product has superior microbial and sensory quality (Savandago *et al*., 2004). The main difference between traditionally fermented milk and industrially produced fermented milk has been attributed to the types of fermenting microorganisms, which produce different types of flavour (Mufandaedza *et al.*, 2006). Therefore, in order to develop a suitable starter culture for industrially fermented milk, isolation and identification of the dominant bacteria involved in the fermentation of traditionally fermented milk products and the use of the isolates as starter cultures is essential.

D. Milk fermenting microbes

Lactic acid bacteria

Lactic acid bacteria (LAB) are non-sporulating, aerotolerant cocci or rods, which produce lactic acid as one of the main fermentation products. According to the current taxonomic classification they belong to the phylum *Firmicutes,* class *Bacilli,* and order *Lactobacillales* (Benniga, 1990; Lathinen *et al.,* 2012*)*. LAB can be divided into two groups based upon the products produced from the fermentation of glucose. Homo-fermentative organisms ferment glucose to two moles of lactic acid, generating a net of 2 ATP per mole of glucose metabolised and lactic acid is the major product. Hetero-fermentative LAB ferment 1 mole of glucose to 1 mole of lactic acid, 1 mole of ethanol and 1 mole of carbon dioxide. One mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized. Because of the low energy yields, LAB often grow slower than microorganisms capable of respiration and produce smaller colonies of 2 - 3 mm (Law, 1997; Varnam & Sutherland, 2001).

LAB have been used to ferment or culture foods for at least 4000 years and fermented milk products from all over the world, including yoghurt, cheese, butter, buttermilk, kefir and koumiss (a mildly alcoholic drink made from fermented mare's milk). Lactic acid also gives

fermented milks their slightly tart taste. Additional characteristic flavours and aromas are often the result of other products produced by LAB. For example, acetaldehyde provides the characteristic aroma of yoghurt, while diacetyl imparts a buttery taste to fermented milks. Additional microorganisms such as yeasts can also be included in the culture to provide unique tastes. Alcohol and carbon dioxide produced by yeasts contribute to the refreshing, frothy taste of kefir, koumiss and leben, fermented milk similar to yoghurt (Jay *et al*., 2005; Schaechter, 2009; Lathinen *et al*., 2012).

Lactobacillus

Lactobacillus is one of the most important genera involved in food microbiology and human nutrition, due to their role in food and feed production and preservation, as well as their probiotic properties (Heredia *et al.*, 2009; Ljungh & Wadstrom, 2009). *Lactobacillus* spp. are Gram-positive, non-motile, rod-shaped organisms and according to their metabolism can be divided into three groups. Group 1 are obligatory homo-fermentatives and includes *L. acidophilus*, *L. delbrueckii*, *L. helveticus* and *L. salivarius*. Group 2 contains facultative hetero-fermentative bacteria and includes *L. casei, L. curvatus, L. plantarum* and *L. sakei*. Lastly, bacteria in Group 3 are obligatory hetero-fermentatives and include *L. brevis, L. buchneri, L. fermentum* and *L. reuteri*. These bacteria are widespread and can be isolated from many plant and animal sources. In humans they are present in the gastrointestinal tract, where they make up a small portion of the gut flora (Varnam & Sutherland, 2001; Hutkins, 2006; Heredia *et al.,* 2009).

Lactobacilli contribute to the flavour of fermented foods by the production of acetaldehyde, hydrogen sulphide (H_2S) and amines (Heredia *et al.,* 2009; Ljungh & Wadstrom, 2009). Lactobacilli was the dominant microflora isolated from 22 samples of kule naoto, the traditionall fermented milk of the masaai people in Kenya (Mathara *et al.,* 2004). *Lactobacillus plantarum* and *L. delbrueckii* subsp*. lactis* have been isolated from traditionally prepared Amasi from South Africa (Beukes *et al*., 2001). A wide variety of other lactobacilli were also isolated from Zimbabwean Amasi including *L. helveticus, L. casei* subsp*. casei* and *L. casei* subsp*. pseudoplantarum* (Gadaga *et al*., 1999; 2000; McMastera *et al*., 2005; Todorov *et al*., 2007). Nono/nunu, traditionally prepared in Nigeria and Ghana, is another example of traditionally fermented milk from which a wide variety of lactobacilli, including *L. brevis*, *L. bulgaricus*, *L. plantarum*, *L. casei* and *L. fermentum* has been isolated (Okonkwo, 2011).

Lactococcus

The genus *Lactococcus* consist of seven distinct species, namely *Lc. lactis*, *Lc. garviae, Lc. piscium, Lc. plantarum, Lc. rafinolactis, Lc. chungangensis* and *Lc. fujiensis*. They are all non-motile, obligatory homo-fermentative, facultative anaerobes with an optimum growth temperature of 30°C. They have distinctive microscopic morphology usually appearing as cocci in pairs or short chains (Hutkins, 2006).

One species in particular, *Lc. lactis,* is an industrially important member of the LAB used widely in the fermentation of dairy products, including sour cream, butter milk and various cheeses such as cheddar. When *Lc. lactis* is added to milk, the bacterium uses lactase to produce ATP from lactose with lactic acid as the by-product of ATP production. The lactic acid curdles the milk that then separates to form curds, which are used to produce cheese and whey. Additionally, lactic acid lowers the pH of the product and preserves it from the growth of unwanted microorganisms. Other metabolic products and enzymes produced by *Lc. lactis* contribute to the more subtle aromas and flavours that distinguish different cheeses (Hui *et al.*, 2004).

Lactococcus lactis subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are commonly used as starter cultures for the commercial production of fermented milks such as Omaere, produced in Namibia (Schutte, 2013) and Amasi from South Africa (Beukes *et al.,* 2001). *Lactococcus* spp. have been isolated from traditionally prepared fermented milks like Fulani from Burkina faso (Savandago *et al*., 2004) and Nunu, a spontaneously fermented yoghurt-like milk product consumed as a staple food commodity in parts of West Africa (Akabanda *et al*., 2010).

Leuconostoc

Leuconostoc is a genus of Gram-positive bacteria, placed within the family of Leuconostocaceae. They are generally ovoid cocci often forming chains (Hui & Khachatourians, 1995). All species of this genus have a hetero-fermentative mode of metabolism and are resistant to the antibiotic vancomycin, a useful characteristic for isolation of these bacteria. For the selective growth of *Leuconostoc* spp., vancomycin is added to deMan, Rogosa and Sharpe Medium (MRS) to create an environment favourable for the growth of the bacteria and eliminates the growth of other LAB (Hui & Khachatourians, 1995). *Leuconostoc* spp. are associated with plants and decaying plant materials. They have been detected in green vegetation and roots and in various fermented vegetable products such as cucumber,

kimchi, cabbage and olives. *Leuconostoc* spp. are also frequent in food of animal origin, including raw milk, dairy products, meat, poultry and fish (Wood & Holzapfel, 1995; Lahtinen *et al.,* 2012). *Leuconostoc* spp. can contribute to off flavours due to diacetyl production in certain alcohols, meats, vegetables and fermented milk products like cheese or yoghurt (Hui & Khachatourians, 1995; Priest & Campbell, 2003). *Leuconostoc* strains are also used as starter cultures, for example, in buttermilk and cheese production when ripening occurs at 15ºC rather than 8ºC (Chandan *et al*., 2008). *Leuconostoc* spp. are often isolated from traditionally fermented milk and has been shown to be the predominant LAB group present in traditionally prepared fermented milk samples collected from various households in South Africa and Namibia (Beukes *et al.,* 2001).

Streptococcus

Streptococci are distinguished from *Leuconostoc* by their strictly homo-fermentative metabolism. These organisms can be isolated from oral cavities of animals, the intestinal tract, skin and any foods that come in contact with these environments (Lahtinen *et al.,* 2012). In this genus only one species, namely *Streptococcus thermophiles* is recognised as safe. *Streptococcus thermophiles* is used as a starter along with one or more LAB strains from the genus *Lactobacillus*. These mixed strain starter cultures are used in various dairy fermentations, including the production of yoghurt, fermented milks and Italian and Swiss-type cheeses (Wood & Holzapfel, 1995; Chandan *et al*., 2008).

Acetic acid bacteria

Acetic acid bacteria (AAB) are Gram-negative, rod-shaped organisms present as single cells, pairs or chains that belong to the *Acetobacteraceae* family. There are twelve main genera which belong to the family Acetobacteraceae, namely *Acetobacter, Gluconacetobacter, Gluconobater, Asaia, Acidomonas, Granulibacter, Ameyamaea, Neoasaia, Kozakia, Saccharibacter, Swaminathania* and *Tanticharoenia* (Hutkins, 2006; Oliver, 2012). During fermentation AAB oxidises sugars or ethanol to produce acetic acid (Hui, 1995; Hutkins, 2006).

AAB are widespread in nature and isolated from flowers, fruits, herbs and cereals. The best known industrial application of AAB is in vinegar production (Oliver, 2012). AAB can cause spoilage in wine, ciders and beer by producing excessive amounts of acetic acid or ethyl acetate (Solieri & Giudici, 2009; Oliver, 2012). However, these bacteria are also used

intentionally to acidify beer during long maturation periods in the production of traditional Flemish Sour Ales (Fungelsang & Edwards, 2007; Oliver, 2012).

Species of AAB have been isolated from dairy products and are used as commercial starter bacteria, for example *Acetobacter orientalis* in combination with *Lc. lactis* subsp. *cremoris* is used as starter culture to produce fermented milk in Japan (Nakasaki *et al*., 2008). This is done to provide or enhance the characteristic flavors and textures of fermented milk (Nakasaki *et al*., 2008). *Acetobacter syzygii* has been isolated from kefir grains obtained from naturally fermented kefir (da Cruz Pedrozo Miguel *et al*., 2010). *Acetobacter aceti, A. lovaniensis, A. orientalis* and *A. pasteurianus* have been isolated from mashita, a traditionally prepared butter fat in Uganda (Ongol & Asano, 2009).

Yeasts

Yeasts are eukaryotes classified as members of the Kingdom Fungi and are aerobic, oval-shaped and slightly larger than bacteria (Querol & Fleet, 2006; Feldmann, 2011). Yeasts are found in soil, water, on the surface of plants, and on the skin of humans and other animals. Like other fungi, yeasts obtain food from the organic matter around them as they secrete enzymes that break down the organic matter into nutrients they can absorb (Hui *et al*., 2004; Feldmann, 2011). In baking, yeast is used as a leavening agent through digesting sugars from the bread dough and producing carbon dioxide. In brewing, yeast digests sugar from malt and produces alcohol and carbon dioxide (Hui *et al*., 2004).

Yeasts have been isolated from various traditionally fermented milks from Africa. Twenty yeast species were isolated from 30 samples of traditionally prepared Zimbabwean Amasi (Gadaga *et al.*, 2000). *Candida krusei, Geotrichum penicillatum* and *Rhodotorula mucilaginosa* have been isolated from 15 samples of Sussac, a Kenyan traditionally fermented camel milk product (Lore *et al.*, 2005). Its presence in dairy products leads to an end-product which differs in physio-chemical properties from those made with pure LAB starters as yeast promotes symbiosis among microorganisms present. These cells form carbon dioxide adding fizziness and contribute to the specific aroma and slight yeasty taste of traditionally fermented milk (Boulton & Quain, 2001; Priest & Campbell, 2003).

E. Antimicrobial activity of lactic acid bacteria

LAB produce antimicrobial components during fermentation that frequently inhibit the growth of pathogens, as well as other spoilage microorganisms. These antimicrobial components include organic acids, hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins. According to Kasra-Kermanshahi & Mobarak-Qamsari (2015) consumers prefer natural foods without chemical preservatives. By incorporating LAB in commercial starter cultures the use of chemical preservatives such as sodium benzoate and sodium metabisulphite can be reduced to ensure food preservation (Naidu, 2000; Toldra, 2009; Lahtinen *et al.,* 2012; Chandan *et al*., 2008).

The survival and growth of *E. coli* 3339 and *Salmonella enteritidis* 949575 were studied in milk fermented with LAB and yeast strains, previously isolated from Zimbabwean naturally fermented milk. The study showed that *Lc. lactis* subsp. *lactis* strongly inhibits the pathogenic *E. coli* and *S. enteritidis* strains tested. The main inhibitory effect seemed to be associated with fast acid production which resulted in rapid pH reduction. Addition of *Candida kefyr* did not have a significant effect on the rate of inhibition (Mufandaedza *et al*., 2006).

Organic acids

The preservative action of starter cultures in food and beverage systems is attributed to the combined action of a range of antimicrobial metabolites produced during the fermentation process. These include many organic acids such as lactic, acetic and propionic acids produced as end-products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms (Theron & Lues, 2010; Lahtinen *et al*., 2012). Acids exert their antimicrobial effect by interfering with the maintenance of the cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions. They have a very broad mode of action and inhibit Gram-positive and Gram-negative bacteria, as well as yeasts and moulds (Chandan *et al*., 2008). In the study by Yang *et al. (*2012) cell free supernatants from eight LAB isolates significantly inhibited the growth of *Listeria innocua, Bacillus cereus*, *Pseudomonas fluorescens*, *Erwinia carotovora,* and *Leuconostoc mesenteroides* subsp*. mesenteroides*.

Hydrogen peroxide

The antimicrobial properties of hydrogen peroxide have been recognised for many years and currently it is widely used in the food industry for aseptic packaging of liquid food products. LAB contains flavoproteins which oxidise to produce hydrogen peroxide in the presence of oxygen (Chandan *et al*., 2008). The absence of the catalase enzyme in LAB leads to sufficient amounts of hydrogen peroxide accumulation in fermented milk to have inhibitory effects (Heredia *et al.,* 2009). The amount of hydrogen peroxide produced depends on the availability of oxygen in the food medium at the beginning of fermentation and the microbial species and strains present (Lahtinen *et al.,* 2012; Ray & Bhunia, 2014).

Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species (narrow spectrum), or across genera (broad spectrum) (Riley & Chavan, 2007). In recent years, bacteriocin producing LAB have attracted significant attention because of their generally recognised as safe (GRAS) status and potential use as additives to ensure food preservation. Nisin, produced by *Lc. lactis* is the most thoroughly studied bacteriocin to date and has been applied as a food additive worldwide (Gould, 1999; Caplice & Fitzgerald, 1999). In the study by Tondorov & Dicks (2006) strains of *L. plantarum*, *L. pentosus*, *L. rhamnosus* and *L. paracasei* isolated from boza (a traditional cereal beverage from Bulgaria) produced bacteriocins active against *E. coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*.

Carbon dioxide

Hetero-fermentative LAB produces carbon dioxide as an end-product of hexose fermentation. The antimicrobial effect of carbon dioxide is achieved in two ways. Firstly, an anaerobic environment is created in the fermented milk product which favours the growth of anaerobic LAB and some yeasts but inhibits obligated aerobic microorganisms such as mycelial fungi and Gram-negative bacteria. Secondly, a rise in the carbon dioxide pressure may result in inefficient cell membrane transport mechanisms, which mediate pH changes of intracellular and extracellular environments and inhibit enzymatic reactions (Lindgren & Dobrogosz, 1990; Adams & Nicolaides, 1997; Caplice & Fitzgerald, 1999).

Diacetyl

Diacetyl produced by hetero-fermentative LAB during fermentation can have antimicrobial effects. Diacetyl (2,3-butanedione), an end-product of citrate metabolism, is important for flavour and aroma formation in dairy products. The buttery aroma and taste are due to diacetyl production by LAB during its production. This compound also inhibits various microorganisms such as *E. coli, Salmonella* spp*., S. aureus, Bacillus* spp*., Mycobacterium tuberculosis* and *Aeromonas hydrophila* (Adams & Nicolaides, 1997; Caplice & Fitzgerald, 1999). The antimicrobial mechanism of diacetyl is active at a low pH and is believed to be the cause of the disruption of arginine utilization. Although diacetyl is a well-known antimicrobial compound, the concentration produced is often too low to have a measurable lethal effect (Adams & Nicolaides, 1997; Caplice & Fitzgerald, 1999). An increase of this component during fermentation to ensure antimicrobial activity can lead to a bitter after-taste in fermented milk (Lindgren & Dobrogosz, 1990; Caplice & Fitzgerald, 1999).

F. African traditionally fermented milk products

In Africa, a wide variety of traditionally prepared fermented milk products are produced at household level in the rural areas. These are prepared by spontaneous fermentation from microorganisms in the environment and those inherent in the raw milk or by back slopping (Hamama, 1992). Fermented milks have a characteristic semi-solid and curdled texture (Tamine, 2006).

Traditional milk fermentation processes have been manipulated by the indigenous people in order to preserve and improve the quality. One such method is the draining of whey (40 - 50%) after fermentation and mixing of the curd to a smooth consistency. The process reduces the volume of the original product as some whey has to be drained off to obtain the desired consistency. Some milk producers smoke the fermenting milk containers and the milk with wood of certain tree species, as a method of improving the flavour, colour, taste and palatability.

Table 1 African traditionally fermented milk products

Milk product	Source	Country	Microbial Genera	Possible pathogens	Reference
Suusac	Bovine	Somalia	*Lactobacillus* *Leuconostoc* *Lactococcus* Yeasts	*Enterococcus*	Lore *et al*., 2005
Ititu	Camel	Ethiopia	*Lactobacillus* *Lactococcus*	*Enterococcus faecalis*	Seifu *et al*., 2012
Ergo	Bovine	Ethiopia	*Lactobacillus* *Leuconostoc* *Streptococcus* *Lactococcus*	*E. faecalis*	Gonfa *et al*., 2001
Kule naoto	Bovine	Kenya	*Lactobacillus* *Lactococcus* *Leuconostoc*	*Enterococcus faecium* *Enterobacteriaceae*	Mathara *et al*., 2004
Amasi/ Mukaka wakora	Bovine	Zimbabwe	*Lactobacillus* *Lactococcus*	Coliforms	Gadanga *et al*., 2000
Raib	Bovine	Morocco	*Lactococcus* *Lactobacillus* *Streptococcus* *Leuconostoc*	*E. faecium* *E. faecalis*	Hamama, 1992; Elotmani *et al*., 2002
Fulani	Bovine/ Caprine	Burkina faso	*Lactobacillus* *Leuconostoc* *Lactococcus* *Streptococcus*	*Enterococcus* *Enterobacteriaceae*	Savadogo *et al.*, 2004
Nunu	Bovine	Ghana	*Lactobacillus* *Leuconostoc* *Lactococcus* *Streptococcus* Yeasts	*Enterobacter* *Klebsiella* *E. coli* *Proteus vulgaris* *Shigella*	Akabanda *et al*., 2010
Amasi	Bovine	South Africa	*Leuconostoc* *Lactococcus* *Lactobacillus*	*S. aureus* *Enterococcus*	Beukes, *et al*.,2001
Omashikwa	Bovine	Namibia	*Lactobacillus* *Lactococcus* *Leuconostoc*	*E. faecium,* *Enterococcus durans* *E. coli* *Staphylococcus* spp.	Bille, 2009; Schutte, 2013

Most traditionally prepared fermented milk in Africa are the results of lactic acid fermentation by LAB, although other microorganism can also be present such as yeasts (Table 1). The quality of this traditionally prepared fermented milk is often poor due to neglected hygienic practices during preparation which leads to the detection of possible pathogens in the final products.

Omaere, a Namibian traditionally fermented bovine milk product

Namibia is a country in Southern Africa where the western border is the Atlantic Ocean. It shares land borders with Angola and Zambia to the north, Botswana to the east and South Africa to the south and east. Milk production has been part of the culture in the communal areas in Namibia for centuries and significantly contributes to the daily nutritional needs of rural communities. Milk fermentation is the main technique used in order to preserve the milk. The commercial Namibian dairy industry is characterised by a single large dairy product manufacturer, Namibia Dairies, which is involved in milk production, manufacturing, as well as distribution to retailers.

Omaere is a traditional Namibian fermented full cream milk product, consumed as a staple food among the Herero community of Namibia. The Herero are traditionally cattle-herding pastoralists who rate status on the number of cattle owned. Omaere is traditionally prepared by spontaneous fermentation of unheated bovine milk in a calabash. The calabash (*Lagenaria siceraria)* is a vine, grown for its fruit which can either be harvested young and used as a vegetable or harvested mature, dried and used as a fermentation container for Omaere. A previously used calabash is used as the starter culture in the production of Omaere at household level. New calabashes need to be seeded with a natural microbial inoculum before it can be used for the production of fermented milk. The calabash gourd is first cleaned using a mixture of water and small stones. Once the calabash is clean, it is filled with fresh cow milk and allowed to ferment naturally for 1 - 2 days at ambient temperature. The milk is discarded and the calabash is filled with fresh milk and this is repeated 3 to 4 times before the milk is used for human consumption. Omaere is white, has a low-viscosity with an acidic taste and is consumed as a weaning food, a refreshing drink or with other food products such as porridge. It is also commercially produced from full cream milk with the addition of non-fat milk solids, sugar (optional) and starter culture. The final product has a less acidic taste and a more creamy texture in comparison to the traditionally fermented Omaere. The LAB isolated from

commercially produced Omaere were *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* (Schutte, 2013).

Figure 2 Example of calabash as a fruit and as fermentation container for Omaere production

G. Conclusion

Modern socio-economic changes can mean some traditional technologies for producing fermented milk will eventually be lost, together with the associated microorganisms. It is, therefore, imperative that the traditional indigenous fermented, as well as the preservation and exploitation of the associated fermentative micro-organisms be scientifically investigated. This can be achieved by isolating and enumerating the microorganisms in traditionally fermented milk and studying their functional properties. On an industrial scale, these microbes can be used to develop new starter cultures to produce fermented milk products with similar aroma, flavour and texture characteristics. In order to meet the demand of consumers for natural food without preservatives, these cultures must be investigated for their antimicrobial activity and used as natural preservatives in food.

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Chapter 3

Isolation, enumeration and identification of microorganisms from Namibian traditionally fermented milk Omaere

Abstract

Traditionally fermented milk is prepared as means of preserving milk against spoilage. Fermentation originally involved microorganisms present in the raw milk. The environment under which the raw milk was placed, addition of indigenous roots to the raw milk and smoking of fermentation containers resulted in conditions that selected for the desirable microorganisms. However, with the development of modern technologies, specific lactic acid producing microorganisms are introduced into pasteurised milk and fermentation is carried out industrially under controlled conditions. In this way, fermented products of superior sensory and microbial qualities are produced. However, use of a limited number of well-defined starter cultures results in very similar products, reducing the diversity of fermented milk products. Therefore, in order to enhance product diversity, isolation and identification of the dominant bacteria involved in the fermentation of traditionally fermented milk products and the use of the isolates as starter cultures, is essential. In this study, the fermenting microorganisms found in five samples of traditionally fermented milk Omaere from Namibia were isolated and enumerated using six different selective media. The only lactic acid bacteria found in Namibian fermented milk Omaere belonged to the genus *Lactobacillus* with the predominant species *Lactobacillus plantarum* (63%) (n=87), followed by *Lactobacillus paracasei* subsp. *paracasei* (15%), *Lactobacillus kefiri* (11%), *Lactobacillus paraplantarum* (9%) and *Lactobacillus casei* (2%). The yeasts isolated were *Kazachstonia unispora* (50%) (n=18), *Saccharomyces cerevisiae* (44%) and *Candida pararugosa* (6%). The *Lactobacillus* spp. isolated from Omaere may be used to develop new and original fermented dairy products with unique tastes, aromas and characteristics which can be produced on an industrial scale.

Introduction

Milk fermentation is the most widely used traditional method of milk preservation. This method increases the shelf-life of the product and improves the taste and digestibility of milk (Patton, 2004). Traditional fermented milk products have been prepared for centuries all over the world.

However, it was not until the days of Louis Pasteur, about 100 years ago, that the microbiology underlying fermentations was revealed. In contrast, industrially fermented milk products were only developed in the 1900s (Kurmann, 1994). These are based on known scientific principles, with specific starter cultures and optimized quality (Kurmann, 1994; Patton, 2004; Tamine, 2009). The microbiological content of fermented milk products have been studied in countries such as Burkina Faso (Savadogo *et al*., 2004), Ethiopia (Gonfa *et al*., 2001), Indonesia (Yodoamijoyo *et al.*, 1983; Hosono *et al.*, 1989), Morocco (Hamama, 1992), Namibia (Bille, 2009), South Africa (Keller & Jordan, 1990; Beukes *et al.*, 2001), Sudan (Abdelgadir *et al*., 1998, 2001) and Zimbabwe (Gadaga *et al*., 2000). The nature of fermented products varies from one region to another and depends on the local indigenous microorganisms, which in turn reflects the climatic conditions of the area. According to Savadogo *et al*. (2004), traditional fermented milk in regions with a colder climate contained mesophilic bacteria such as *Lactoccoccus* and *Leuconostoc* spp., whilst thermophilic bacteria such as *Lactobacillus* and *Streptococcus* spp. prevailed in regions with a hot or subtropical or tropical climate.

Omaere is traditionally fermented bovine milk kept by the Herero community of Namibia. This is a household product which results from natural, uncontrolled fermentation of untreated raw milk in a calabash. The calabash is a vine (*Lagenaria siceraria*) grown for its fruit which can either be harvested young and used as a vegetable or harvested mature, dried and used as a fermentation container for Omaere. The fermentation process occurs spontaneously by back-sloping during which fresh milk is added to a calabash that was previously used to produce Omaere. A new calabash is inoculated by allowing milk to ferment naturally for 1-2 days, the milk is than discarded and the calabash is filled with fresh milk and the process is repeated 3 to 4 times before the milk is used for human consumption. The microorganisms in the walls of the calabash and present in the milk are used as the starter culture. The fermented product is used for quenching thirst or as a condiment to porridge.

Bacteria found in traditional fermented milk represent a unique genetic resource for innovative food biotechnology. It is, therefore, important that traditional indigenous Omaere, as well as the preservation and exploitation of the associated fermentative microorganisms be scientifically investigated (Kurmann, 1994). The aim of this study was to isolate, enumerate and identify microorganisms from Namibian traditional fermented milk Omaere collected from different regions in Namibia.

Materials and methods

Sample collection

A representative sample of traditionally fermented Omaere was obtained by sourcing five samples from households in different regions of Namibia where the Herero community resides (Fig. 3). According to the climate data for December 2012, Otjozondjupa, Omaheke, Khomas and Erongo regions were the samples were sourced had a similar annual temperature range of between 14°C and 32°C. The samples were collected in sterile 50 ml bottles, labelled, cooled on ice and transported to the laboratory. The pH of each sample was measured on arrival at the lab using a pH meter (Crison).

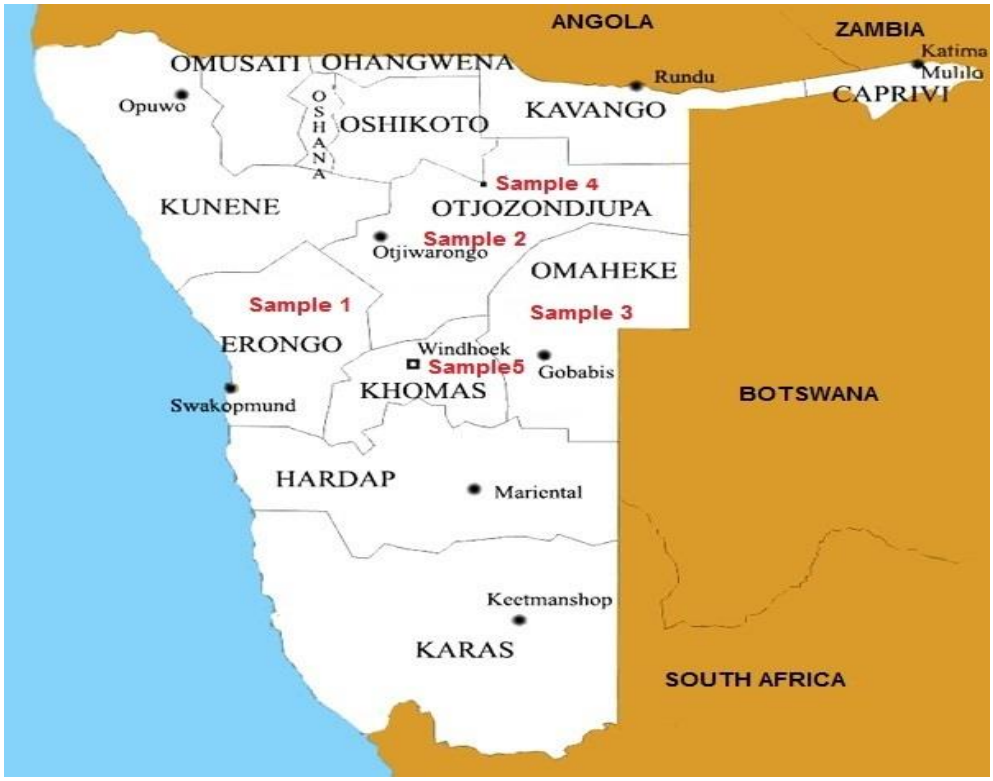

Figure 3 Regions where Omaere samples were sourced in Namibia.

Isolation and enumeration

Each fermented milk sample was mixed with a vortex to ensure homogenization of the microorganisms present in the fermented milk. One ml of each fermented milk sample was diluted in sterile saline solution (0.85% (w/v) NaCl, Merck) and a dilution series to 10^{-6} was

prepared in duplicate. One ml of each of these serial dilutions was pipetted into appropriately marked petri dishes. This was done for six different selective media (Table 2) which was pour plated into these petri dishes and properly mixed for 30 s. The media that were used for the isolation and enumeration of *Lactobacillus* spp. (MRS+C) and *Leuconostoc* spp. (MRS+V) were incubated anaerobically in 3 L glass bottles (Consol) in the presence of a gas-generating kit (Anaerocult A system, Merck) at 30°C for 3 to 5 days. The media that were used for the isolation of yeasts (CGA and PDA) were incubated aerobically at 25°C for 3 to 5 days. Media used for the isolation of *Lactococci* spp. (M17 agar) and acetic acid bacteria (AAB) and (MRS+E) were incubated aerobically at 30°C for 3 to 5 days. The viable microbial counts of the bacteria and yeasts suspended in each serial dilution were determined and expressed in colony forming units per millilitre (cfu.ml^{-1}) of fermented milk.

Table 2 Growth media used for the isolation and enumeration of microorganisms in Omaere

Media	**Composition**	**Microorganism**
MRS+C	deMan, Rogosa and Sharpe Medium (MRS) with 100 µg.ml^{-1} cycloheximide (Sigma) soluble in ethanol (Merck) (stock solution concentration of 50 mg.ml^{-1}) added to the MRS-medium after sterilisation (Pintado *et al*., 1996).	*Lactobacillus* spp.
MRS+V	deMan, Rogosa and Sharpe Medium (MRS) with 30 µg.ml^{-1} vancomycin (Sigma) diluted in dd$H_2$0 (stock solution concentration of 50 mg.ml^{-1}) added to the MRS medium after sterilisation (Benkerroum *et al*., 1993).	*Leuconostoc* spp.
MRS+E	deMan, Rogosa and Sharpe Medium (MRS) (Merck) with 2% (v/v) ethanol (Merck).	Acetic acid bacteria
M17	M17 agar (Merck) Prepared according to manufacture specifications.	*Lactococci* spp.
CGA	Chloramphenicol Glucose Agar (Merck) prepared according to manufacture specifications.	Yeasts
PDA	Potato Dextrose Agar (Merck) prepared according to manufacture specifications.	Yeasts

Strain selection and cultivation

The number of bacterial and yeast colonies selected from specific media plates for further identification was determined by means of the Harrison Disc method (Harrigan, 1998). This method allows selection of multiple colonies from a single media plate representing the predominant microbial group or groups in the fermented milk for identification (Harrigan, 1998). The selected colonies were streaked out on media plates used for cultivation in order to obtain pure cultures. Lactic acid bacteria (LAB) colonies selected from MRS+C, MRS+V, M17 agar and AAB selected from MRS+E were streaked on MRS agar with no supplements and incubated aerobically at 30°C for 2 days. Yeast colonies from both PDA and CGA were streaked on PDA and incubated aerobically at 30°C for 2 days. The obtained pure cultures were used for DNA extraction.

DNA extraction

Bacterial colonies were inoculated in 5 ml MRS broth and incubated overnight at 37°C. DNA of the selected bacterial colonies was extracted using 2 ml of the overnight culture pipetted into a 2 ml eppendorf tube and centrifuged at 16000 x g for 5 min to concentrate the bacterial cells in a pellet. The supernatants were discarded and the pellets were suspended in 1 ml of 2 X saline sodium citrate (SSC) (Sigma) and sodium dodecyl sulphate (SDS) (Sigma). The mixture was then heated to 99°C for 10 min. Cells were collected by centrifugation at 16000 x g for 5 min and the supernatant was discarded. One ml of deionized water was then added to the cell pellet and centrifuged at 16000 x g for 5 min. The supernatant was discarded and 200 µl glass beads, 500 µl chloroform, 200 µl of TE buffer and RNase (Qiagen) was added to the cell pellet. The mixture was then vortexed for 10 min and centrifuged at 16000 x g for 15 min. The supernatant was transferred to a new tube and used directly as template for PCR amplification (Xiao, 2006).

Whole cell PCR was performed on the selected yeasts colonies. Cells were added to 25 µl of deionized water using a sterile tip. The mixture was then incubated at 96°C for 10 min and after cooling, it was used directly as template for PCR amplification (Luo & Mitchell, 2002).

PCR amplification

The primers 27F and R1492 (Frank *et al*., 2008) were used to amplify a 1.5 kilobase (kb) fragment of the 16S ribosomal RNA (rRNA) gene of the selected bacterial colonies. The final

concentrations of reagents in the PCR reaction mixture (25 µl total volume) were 0.4µM of both primers 27F and R1492, 3.96% (v/v) DMSO (Merck), 0.2 mM dNTPs (Fermentas), 5 U Taq DNA polymerase (New England Biolabs), 1 X buffer (New England Biolabs) containing 10mM TrisHCl, 50mM KCl, pH 8.3 (at 25ºC) and 1.5mM $MgCl_2$, 10 - 40 ng DNA template and 1µl ddH_2O. Amplification parameters of the PCR reaction were initial denaturation at 92ºC for 3 min, followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s, elongation at 68°C for 60 s and a final elongation step at 72°C for 7 min (Singleton, 2000).

The primers ITS4 and ITS5 (Jackson *et al*., 1999) were used to amplify a 600 base pairs fragment of the internal transcribed spacer (ITS) regions of fungal ribosomal DNA (rDNA) and NL1 and NL4 (O`Donell, 1992) were used to amplify the D1/D2 domain of the 26S rRNA gene of the selected yeast isolates. Amplification parameters of the PCR reaction were initial denaturation at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 60 s and a final elongation at 72°C for 10 min (O`Donell, 1992; Jackson *et al*., 1999).

The successful PCR amplification products were cleaned up prior to sequencing in a mixture consisting of 5 µl PCR mixture, 0.5 µl exonuclease and 1 µl of FastAP thermo sensitive alkaline phosphatase (Thermo Scientific). The mixture was incubated at 37°C for 15 min and the reaction was stopped by heating the mixture to 85°C for 15 min (Joos & Fortina, 2005). The clean PCR product was used for DNA sequencing.

DNA sequencing and strain identification

The cleaned PCR product was sequenced using the Big Dye terminator v.3.1 sequencing kit in a 10 µl reaction consisting of 1x buffer, 0.1 x premix, 0.32 pmol 27F, 10 - 40 ng PCR product and 4.5 µl ddH_2O (Kieleczawa, 2005). In the alternative reaction the 27F primer was replaced with 0.32 pmol R1492. The resulting product was cleaned by adjusting the volume to 20 µl with ddH_2O and transferred to a 1.5 ml eppendorf tube that contained 5 µl 125 mM EDTA (Merck) and 60 µl absolute ethanol (Merck). The mixture was then vortexed for 5 s to mix and precipitated at room temperature for 15 min, followed by centrifugation at 4°C for 15 min at 20 000 x g. The supernatant was completely aspirated and 200 µl of 70% ethanol was added to the tubes which were then centrifuged at 4°C for 5 min at 20 000 x g (Liu, 2010). The supernatant was completely aspirated and the resulting pellet was dried in a speed-Vac Concentrator (Eppendorf) for 5 min and sequenced using an ABI 3130xl Genetic Analyser.

Sequences obtained were compared to sequences listed in the NCBI database using the BLAST algorithm and identified according to the closest relative (Edwards *et al*., 2009).

Statistical analysis

Statistical analysis was done using Microsoft excel, 2010. Due to the abnormal nature of the data all enumeration values analysed were transformed to log10. A one way ANOVA was performed on enumeration values (duplicate means) obtained for each sample per medium. If the P (P1) value obtained was significant (P1 value < 0.05) it indicated that at least one of the sample enumeration values differed from another per medium. In that case a two way ANOVA was performed to determine which samples differed significantly (P2<0.05) from each other per medium.

Results and discussion

The pH of fermented milk is a measure of the molar concentration of hydrogen ions in the product and as such is a measure of the acidity or basicity of the fermented milk (Prichard, 2003). The pH value recorded in the traditionally fermented Omaere samples analysed in this study ranged from 3.22 - 3.77. These values are slightly lower than those reported from industrially produced Omaere with an average pH of 4.2 (Schutte, 2013) and Amasi, a South African fermented milk with an average pH value of 4.5 (Beukes *et al.,* 2001). However, traditionally fermented Omaere is more comparable in terms of pH to Nunu, fermented milk in Ghana with a pH value of 3.4 (Akabanda *et al.*, 2010) and Omashikwa, a Namibian traditionally fermented buttermilk with a pH value of 3.3 (Bille *et al*., 2007).

Microbial enumeration

The highest microbial count obtained from Omaere samples 2 and 3 were from MRS+V (3.9 x 10^7 and 5.5 x 10^7 cfu.ml^{-1}) used for the growth of *Leuconostoc* spp., while the highest values for Omaere samples 1, 4 and 5 were obtained from MRS+C, used for the growth of *Lactobacillus* spp. (3.1 x 10^6, 2.0 x $10^7$and 1.5 x 10^7 cfu.ml^{-1}). However, significant statistical differences were observed using a one way ANOVA test between all samples on all media used (P1 < 0.05). Therefore the one way ANOVA was followed by a two way ANOVA to determine which samples enumeration values differed significantly from each other on each medium. On MRS+E used

for the isolation of AAB, sample 5 enumeration value differed significantly from all the other samples (P2 = 0.02, 0.02, 0.00 and 0.02 < 0.05). Sample 1 enumeration value did not differ significantly from sample 4 enumeration value (P2 = 0.8 > 0.05) but differed significantly from samples 2 and 3 enumeration values (P2 = 0.02 and 0.02 < 0.05). Sample 2 enumeration value did not differ significantly from samples 3 and 4 enumeration values (P2 = 0.08 and 0.09 > 0.05) but samples 3 and 4 enumeration values differed significantly from each other (P2 = 0.02 < 0.05). On MRS+C used for the isolation of *Lactobacillus* spp., sample 1 enumeration value differed significantly from samples 2 and 3 enumeration values (P2 = 0.03 and 0.03 < 0.05) but did not differ significantly from samples 4 and 5 enumeration values (P2 = 0.06 and 0.06 > 0.05). Samples 2, 4, and 5 did not differ significantly from each other (P2 = 0.67, 0.30 and 0.13 > 0.05) but differed significantly from sample 3 enumeration value (P2 = 0.03, 0.48 and 0.03 < 0.05). This similarity between samples 2, 4 and 5 can also be observed in pH 3.50, 3.22 and 3.22 respectively, compared to sample 1 (pH = 3.64) and sample 3 (pH = 3.77). This suggests that pH plays an important role in the growth of *Lactobacillus* spp. On M17 used for the isolation of *Lactococci* spp., sample 3 enumeration value did not differ significantly from samples 4 and 5 enumeration values (P2 = 0.06 and 0.053 > 0.05) but differed significantly from samples 1 and 2 enumeration values (P2 = 0.00 and 0.02 < 0.05). Samples 1 and 4 enumeration values did not differ significantly (P2 = 0.62 > 0.05) and from samples 2 and 5 enumeration values (P2 = 0.07, 0.06, 0.09 and 0.08 > 0.05) but samples 2 and 5 enumeration values differed significantly (P2 = 0.01 < 0.05). On MRS+V used for the isolation of *Leuconostoc* spp. sample 1 enumeration values differed significantly from all the other samples enumeration values (P2 = 0.00 , 0.00 , 0.01 and 0.03 < 0.05). Samples 2 and 3 enumeration values did not differ significantly (P2 = 0.07 > 0.05) but differed significantly from samples 4 and 5 enumeration values (0.03, 0.04, 0.04 and 0.048 < 0.05). Samples 4 and 5 enumeration values did not differ significantly (P2 = 0.09 > 0.05). For the yeasts enumeration, Samples 1, 4 and 5 enumeration values did not differ significantly (P2 = 0.13, 0.87 and 0.06 > 0.05) but differed significantly from samples 2 and 3 enumeration values (P2 = 0.00,0.00,0.00,0.04,0.01 and 0.04 < 0.05). Samples 2 and 3 enumeration values differed significantly (P2 = 0.00 < 0.05).

This variances between the studied Omaere sample enumeration values on each medium can be attributed to the inconsistency in preparation of traditionally fermented Omaere, the possibility of exposure of the raw milk to varying environmental conditions such as temperature changes and hygiene standards, influencing the composition and growth of the microbial population present. The Omaere samples used in this study were sourced from different

household and different livestock, thus variations in microbial composition of the raw milks may have also resulted in the observed differences in LAB and yeast counts in the fermented products. This inconsistency in production results in products with varying numbers of microorganisms. Therefore, before the microbial population of traditionally fermented Omaere can be characterised, further identification of microorganisms obtained from each medium is important.

Table 3 Enumeration values (cfu.ml^{-1}) obtained for Omaere

Selective media[a]	**Sample 1**[b]	**Sample 2**[b]	**Sample 3**[b]	**Sample 4**[b]	**Sample 5**[b]	**P1 value**[c]
CGA	1.5×10^5	1.4×10^3	5.4×10^4	3.0×10^5	1.5×10^5	0.00
PDA	1.3×10^6	2.1×10^3	7.3×10^4	1.6×10^6	1.2×10^6	0.00
MRS+ C	3.1×10^6	2.1×10^7	4.1×10^7	2.0×10^7	1.9×10^7	0.00
MRS+ V	2.0×10^6	3.9×10^7	5.5×10^7	1.8×10^7	1.5×10^7	0.00
MRS+ E	1.3×10^5	8.0×10^6	2.7×10^7	1.3×10^6	8.7×10^4	0.00
M17	1.3×10^6	7.3×10^6	1.3×10^3	5.5×10^5	6.4×10^4	0.00

[a] The following abbreviations were used: CGA = Chloramphenicol Glucose Agar, PDA = Potato Dextrose Agar, MRS = deMan, Rogosa and Sharpe Medium, C = cycloheximide, V = vancomycin, E = ethanol, M17 = M17 agar.

[b] Average enumeration value obtained from duplicate media plates.

[c] P1 value < 0.05 indicates that at least one of the mean values differs significantly from another using one way ANOVA test.

Bacteria identification

Despite the use of six different selective media for the isolation of different LAB strains, all bacterial isolates that were identified belonged to the genus *Lactobacillus*. This suggests that the fermentation conditions of Omaere allowed for the selection of *Lactobacillus* spp. and the fact that enumerations were observed on all media can be due to low selectivity of the media. The low selectivity of media suggests that the isolated *Lactobacillus* strains were resistant to the antibiotics or that the concentration of the antibiotics added to deMan, Rogosa and Sharpe Medium were too low to have a lethal effect (Atlas, 2006). This clearly shows that it is very

important to take the selectivity of media into account when conclusions are made on the microbial content of fermented milks.

The dominant *Lactobacillus* spp. isolated from sample 1, 2, and 3 (Fig. 4, 5, 6) were *Lactobacillus plantarum,* suggesting that these samples probably had similar fermentation conditions that favoured the growth of *L. plantarum*. These samples also had similar pH values (3.64, 3.50 and 3.77) in comparison to sample 4 and 5 with the same pH value of 3.22. *Lactobacillus plantarum* strains have been isolated from other traditionally fermented milk products such as kule naoto from Kenya (Mathara *et al.*, 2004) and Amasi produced in South Africa and Zimbabwe (Matukumira, 1996; Beukes *et al*., 2001). This is an important species in the fermentation of various plant products and in some dairy products. Some strains are known to produce antimicrobial substances, e.g., plantaricins that are active against certain pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Hurbert & Bringel, 1996). This LAB species is usually found in vegetative environments and is commonly isolated from decaying and fermenting plant materials (Hui & Khachatourians, 1995). Its detection in Omaere may be related to the fact that the fermentation of the milk is carried out in a plant based calabash and to the adaptation of the particular *L. plantarum* strains to the fermentation condition of Omaere.

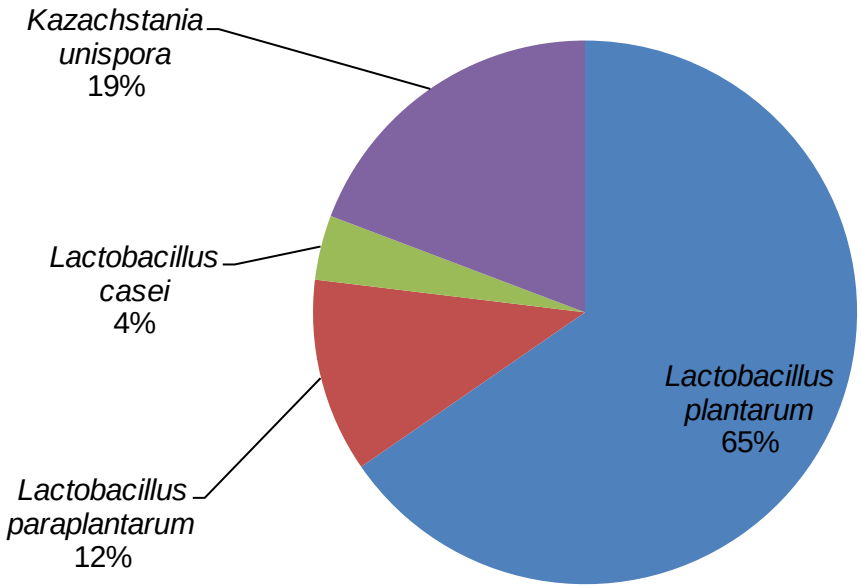

Figure 4 Distribution frequency of prevalent microorganisms in sample 1 (n = 26).

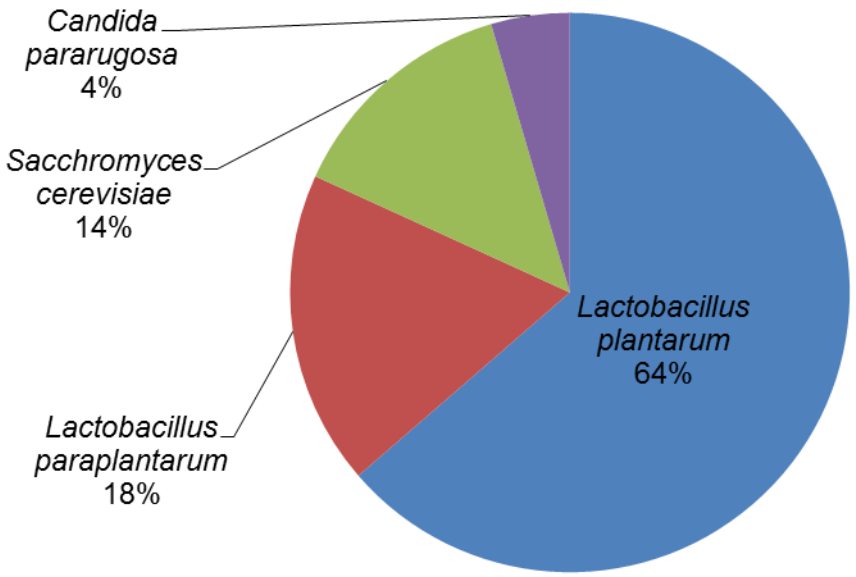

Figure 5 Distribution frequency of prevalent microorganisms in sample 2 (n = 22).

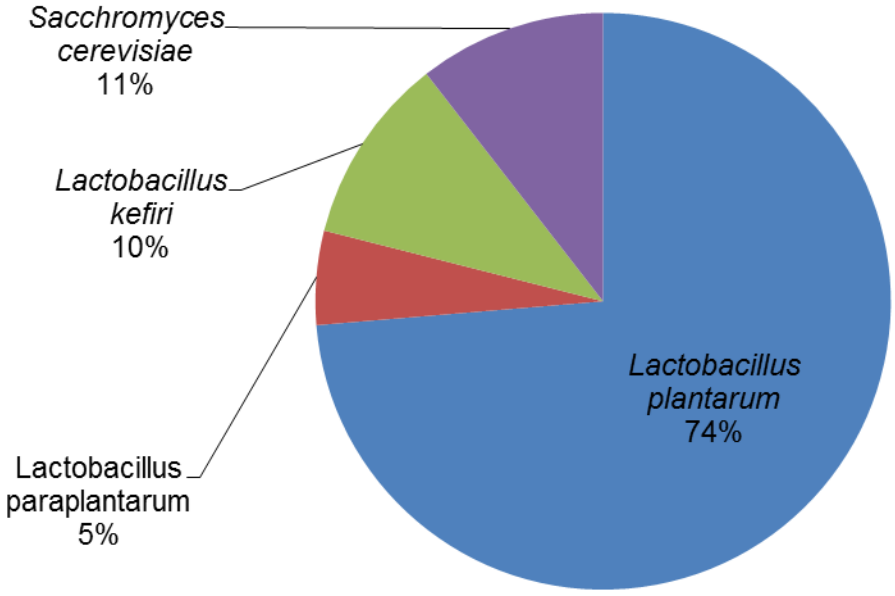

Figure 6 Distribution frequency of prevalent microorganisms in sample 3 (n = 19).

Lactobacillus kefiri was the dominant *Lactobacillus* spp. isolated from sample 4 (Fig. 7). This species is one of the most predominant present in kefir. Kefir is a fermented milk product that is produced by adding kefir grains to milk (Witthuhn *et al.*, 2005). *Lactobacillus kefiri* was also found in naturally fermented Ricotta forte cheese (Baruzzi *et al.*, 2000). Since kefir grains are not used in the production of Omaere, its presence can be due to the adaptation of that strain to the fermentation temperature and pH of Omaere. Therefore, further studies are necessary to determine its origin and role during the fermentation of Omaere.

The dominant *Lactobacillus* spp. isolated from sample 5 was identified as *Lactobacillus paracasei* subsp. *paracasei* (Fig. 8). This species is mostly found in human habitats, sewage and silage and is commonly used as starter culture in dairy product fermentations (Rai & Chikindas, 2011). *Lactobacillus paracasei* subsp. *paracasei* has been isolated by various researchers from fermented milk. This species was the predominant *Lactobacillus* spp. isolated from Garris, a Sudanese fermented camel milk product (Sulieman *et al*., 2006). LAB isolated from Bushera, a Ugandan traditional fermented beverage was identified as *L. paracasei* subsp. *paracasei* (Muyanja *et al*., 2003). This suggests that sample 5 of Omaere had similar fermentation conditions such as temperature with Garris and Bushera that favoured the growth of *L. paracasei* subsp. *paracasei.*

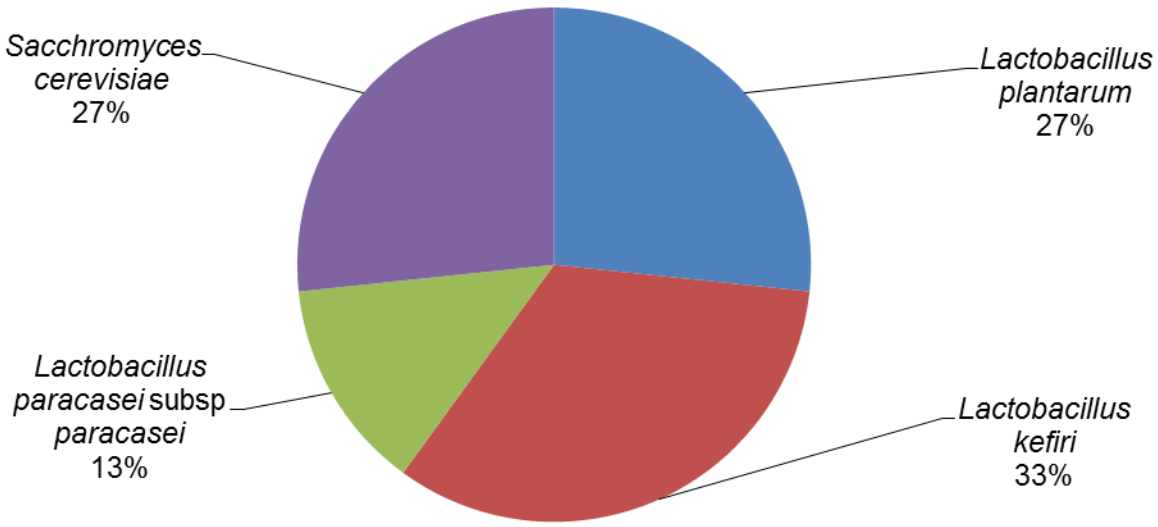

Figure 7 Distribution frequency of prevalent microorganisms in sample 4 (n = 15)

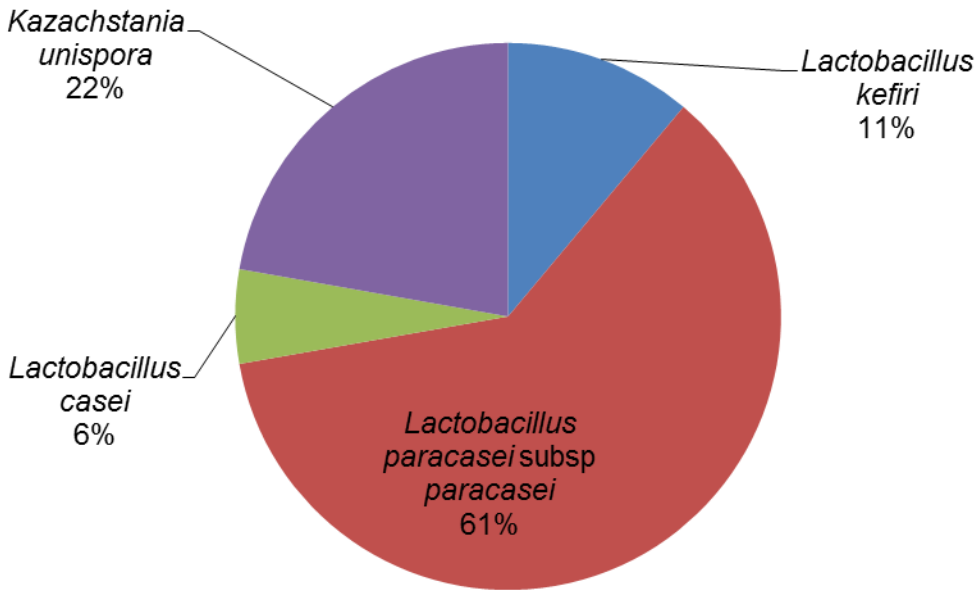

Figure 8 Distribution frequency of prevalent microorganisms in sample 5 (n = 18)

In total 87 bacterial isolates were isolated from the 5 Omaere samples and identified as *L. plantarum* (63%), *L. paracasei* subsp. *paracasei* (15%), *L. kefiri* (11%), *L. paraplantarum* (9%) and *L. casei (*2%) (Table 4). Studies on the microbiological characteristics of several traditionally fermented milk products all reported more than one genus of LAB (Keller & Jordan, 1990; Beukes *et al.*, 2001; Bille, 2009; Hamama, 1992; Savadogo *et al*., 2004; Gonfa *et al*., 2001; Gadaga *et al*., 2000; Abdelgadir *et al*., 1998, 2001). This result supports the theory that the microorganisms that are found in traditionally fermented milks depend on the particular climatic region and the distribution of LAB depends on the nature of fermented milk or fermented food (Savadogo *et al*., 2004). The dominance of thermophilic bacteria in the samples could be explained by the fact that the samples were collected in December 2012 and the ambient temperatures at which the natural fermentation took place were high (32°C).

Table 4 Identification of the microbial strains isolated from Omaere.

Isolate number	Species Identification	%DNA sequence similarity
1 MRS+C 1	*Lactobacillus plantarum*	100%
1 MRS+C 2	*Lactobacillus plantarum*	100%
1 MRS+C 3	*Lactobacillus plantarum*	100%
1 MRS+C 4	*Lactobacillus plantarum*	100%
1 MRS+C 7	*Lactobacillus plantarum*	100%
1 MRS+C 8	*Lactobacillus paraplantarum*	100%
2 MRS+C 1	*Lactobacillus plantarum*	100%
2 MRS+C 3	*Lactobacillus plantarum*	100%
2 MRS+C 4	*Lactobacillus plantarum*	100%
2 MRS+C 7	*Lactobacillus plantarum*	100%
2 MRS+C 8	*Lactobacillus plantarum*	100%
2 MRS+C 11	*Lactobacillus plantarum*	100%
2 MRS+C 12	*Lactobacillus plantarum*	100%
3 MRS+C 1	*Lactobacillus plantarum*	100%
3 MRS+C 2	*Lactobacillus plantarum*	100%
3 MRS+C 3	*Lactobacillus paraplantarum*	99%
3 MRS+C 4	*Lactobacillus plantarum*	100%
3 MRS+C 5	*Lactobacillus plantarum*	100%
3 MRS+C 6	*Lactobacillus plantarum*	100%
4 MRS+C 1	*Lactobacillus kefiri*	100%
4 MRS+C 2	*Lactobacillus kefiri*	100%
4 MRS+C 3	*Lactobacillus kefiri*	100%
4 MRS+C 4	*Lactobacillus kefiri*	100%
4 MRS+C 5	*Lactobacillus kefiri*	100%
5 MRS+C 2	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+C 3	*Lactobacillus kefiri*	100%
5 MRS+C 5	*Lactobacillus kefiri*	100%
1 MRS+V 1	*Lactobacillus paraplantarum*	100%

1 MRS+V 3	*Lactobacillus plantarum*	100%
1 MRS+V 4	*Lactobacillus plantarum*	100%
1 MRS+V 6	*Lactobacillus casei*	100%
2 MRS+V 1	*Lactobacillus plantarum*	100%
2 MRS+V 2	*Lactobacillus paraplantarum*	100%
2 MRS+V 4	*Lactobacillus plantarum*	100%
2 MRS+V 6	*Lactobacillus plantarum*	100%
3 MRS+V 1	*Lactobacillus plantarum*	100%
3 MRS+V 2	*Lactobacillus plantarum*	100%
3 MRS+V 4	*Lactobacillus kefiri*	100%
3 MRS+V 5	*Lactobacillus kefiri*	100%
5 MRS+V 2	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+V 4	*Lactobacillus paracasei* subsp. *paracasei*	99%
1 M17 1	*Lactobacillus plantarum*	100%
1 M17 2	*Lactobacillus plantarum*	100%
1 M17 3	*Lactobacillus plantarum*	100%
1 M17 5	*Lactobacillus plantarum*	100%
1 M17 6	*Lactobacillus paraplantarum*	100%
2 M17 1	*Lactobacillus plantarum*	100%
2 M17 4	*Lactobacillus plantarum*	100%
2 M17 8	*Lactobacillus plantarum*	100%
2 M17 9	*Lactobacillus plantarum*	100%
3 M17 2	*Lactobacillus plantarum*	100%
3 M17 3	*Lactobacillus plantarum*	100%
3 M17 4	*Lactobacillus plantarum*	100%
4 M17 3	*Lactobacillus plantarum*	100%
4 M17 5	*Lactobacillus plantarum*	100%
5 M17 2	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 M17 6	*Lactobacillus paracasei* subsp. *paracasei*	100%
1 MRS+E 1	*Lactobacillus plantarum*	100%
1 MRS+E 2	*Lactobacillus plantarum*	100%
1 MRS+E 3	*Lactobacillus plantarum*	100%
1 MRS+E 5	*Lactobacillus plantarum*	100%
1 MRS+E 6	*Lactobacillus plantarum*	100%

1 MRS+E 7	*Lactobacillus plantarum*	100%
2 MRS+E 1	*Lactobacillus plantarum*	100%
2 MRS+E 2	*Lactobacillus plantarum*	100%
2 MRS+E 3	*Lactobacillus plantarum*	100%
2 MRS+E 4	*Lactobacillus plantarum*	100%
2 MRS+E 5	*Lactobacillus plantarum*	100%
2 MRS+E 6	*Lactobacillus paraplantarum*	100%
2 MRS+E 7	*Lactobacillus paraplantarum*	100%
2 MRS+E 8	*Lactobacillus plantarum*	100%
2 MRS+E 10	*Lactobacillus paraplantarum*	99%
3 MRS+E 1	*Lactobacillus plantarum*	100%
3 MRS+E 2	*Lactobacillus plantarum*	100%
3 MRS+E 3	*Lactobacillus plantarum*	100%
3 MRS+E 4	*Lactobacillus plantarum*	100%
4 MRS+E 2	*Lactobacillus plantarum*	100%
4 MRS+E 3	*Lactobacillus plantarum*	100%
4 MRS+E 5	*Lactobacillus paracasei* subsp. *paracasei*	100%
4 MRS+E 6	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+E 1	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+E 2	*Lactobacillus casei*	100%
5 MRS+E 3	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+E 4	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+E 5	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+E 6	*Lactobacillus paracasei* subsp. *paracasei*	100%
5 MRS+E 7	*Lactobacillus paracasei* subsp. *paracasei*	100%

Yeasts identification

A 0.6 kilobase (kb) fragment of the internal transcribed spacer (ITS) regions of fungal ribosomal DNA (rDNA) of the selected isolates was successfully amplified and sequenced. However, the Blast result was non-specific and therefore the D1/D2 domain was also amplified and sequenced and give more specific results which were used to identify the studied yeast isolates.

The yeast isolates from the Omaere samples in this study were identified as *Kazachstonia unispora*, formerly known as *Sacchromyces unisporus* (50%), *Saccharomyces*

cerevisiae (44%) and *Candida pararugosa* (6%) (Fig. 9). *Kazachstonia unispora* is considered to be a potential producer of farnesol which controls filamentation of pathogenic microorganisms (Bhattacharya *et al.,* 2013). This yeast also produces certain omega unsaturated fatty acids which combat diseases (Bhattacharya *et al.,* 2013). *Kazachstania unispora* and *S. cerevisiae* were the dominant species present in Koumiss a slightly alcoholic fermented mare's milk beverage in China (Mu *et al*., 2012). *Saccharomyces cerevisiae* was the predominant species isolated from 30 samples of the Zimbabwean traditional fermented milk, Amasi (Gadaga *et al*., 2000). The normal habitat of *Candida* spp. is the mucosal membranes and the skin surface of humans and other warm-blooded animals (d'Enfert & Hube, 2007). Its presence in Omaere could indicate poor hygiene and ineffective cleaning procedures and show the need for improved sanitization procedures.

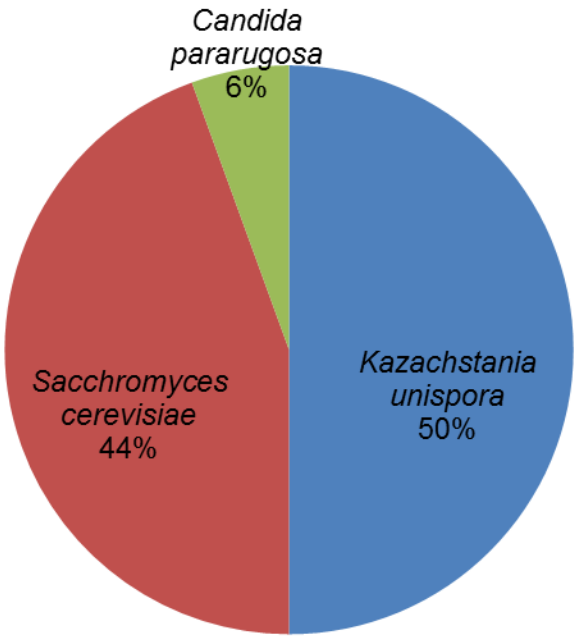

Figure 9 Distribution frequencies of the prevalent yeast species in Namibian fermented milk Omaere (n = 18).

Table 5 Identification of the yeast strains isolated from Namibian fermented milk Omaere

Isolate number	Species Identification	% DNA sequence similarity
1Y1	*Kazachstonia unispora*	100%
1Y3	*Kazachstonia unispora*	100%
1Y4	*Kazachstonia unispora*	100%
1Y5	*Kazachstonia unispora*	100%
1Y6	*Kazachstonia unispora*	100%
2Y1	*Saccharomyces cerevisiae*	100%
2Y2	*Candida pararugosa*	100%
2Y3	*Saccharomyces cerevisiae*	100%
2Y4	*Saccharomyces cerevisiae*	100%
3Y2	*Saccharomyces cerevisiae*	100%
3Y3	*Saccharomyces cerevisiae*	100%
4Y4	*Saccharomyces cerevisiae*	100%
4Y5	*Saccharomyces cerevisiae*	100%
4Y6	*Saccharomyces cerevisiae*	100%
5Y1	*Kazachstonia unispora*	100%
5Y2	*Kazachstonia unispora*	100%
5Y3	*Kazachstonia unispora*	100%
5Y4	*Kazachstonia unispora*	100%

Conclusion

In this study, the LAB and yeast responsible for the spontaneous fermentation of the traditional fermented milk Omaere were isolated and identified. The bacterial isolates that were identified from the Omaere samples tested belonged to the genus *Lactobacillus*. It was established that the media used had low selectivity allowing the growth of *Lactobacillus* spp. on all media used. The *Lactobacillus* and yeast strains isolated from the traditionally fermented milk Omaere can be used to develop new commercial starters. These starters may be used in the production of fermented dairy products with unique tastes, aromas and characteristics on an industrial scale.

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Chapter 4

Milk fermentation by selected single strains of lactic acid bacteria isolated from Omaere and its effect on the survival of *Listeria monocytogenes* and *Escherichia coli*

Abstract

In order to meet the demand of consumers for natural foods without chemical preservatives, alternative methods for controlling pathogenic bacteria in foods should be considered. One practical approach is to incorporate antimicrobial producing cultures into food. In dairy science this can be achieved by the isolation of lactic acid bacteria (LAB) from traditionally fermented milk, studying their antimicrobial activity and possibly incorporate them into fermented products as starter cultures.

Pure cultures of four species of *Lactobacillus* previously isolated from Namibian traditionally fermented milk Omaere, were used to ferment double pasteurised fresh milk inoculated with *Listeria monocytog*enes and *Escherichia coli* with a final concentration of approximately 5 log cfu.ml^{-1} and the interaction was monitored over time. After 48 h of fermentation, *L. monocytogenes* was not detected in milk samples inoculated with *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus casei* subsp. *paracasei*. In contrast, the concentration of *L. monocytog*enes was reduced by 4 log cfu.ml^{-1} after 48 h and not detected after 72 h of fermentation with *Lactobacillus kefiri*. In pasteurised milk fermented without the addition of starter the concentration of *L. monocytogenes* was only reduced by 2.8 log cfu.ml^{-1} after 72 h of fermentation. This suggests that *L. monocytogenes* was sensitive to milk fermentation with the studied *Lactobacillus* spp. and this sensitivity varied according to the time of fermentation.

In milk with *L. plantarum*, *L. paraplantarum* and *L. casei* subsp*. paracasei* after 48 h of fermentation the *E. coli* concentration was reduced by 4 log cfu.ml^{-1} and after 72 h of fermentation no *E. coli* was detected. However, fermentation with *L. kefiri* after 48 h the concentration was decreased by 1 log cfu.ml^{-1} and at the end of the 72 h the *E. coli* concentration was reduced by 1.7 log cfu.ml^{-1}. In milk fermented without the addition of starters the concentration of *E. coli* was only reduced by 1.6 log cfu.ml^{-1} after 72 h of fermentation. Based on these result it was concluded that *E. coli* was sensitive to milk fermentation in the

presence of *L. plantarum*, *L. paraplantarum* and *L. casei* subsp*. paracasei* but not sensitive to *L. kefiri*.

On the basis of this study *L. plantarum*, *L. paraplantarum* and *L. casei* subsp*. paracasei* cultures used in this study can be recommended to be used as starter cultures for the development of new, safe and original commercial fermented dairy products with unique tastes, aromas and characteristics. However, the *L. kefiri* culture is likely not to be used as starter culture as it took longer to eliminate or failed to eliminate the food pathogens used in the study.

Introduction

Fermentation is the oldest method of milk preservation. However, despite the low pH in fermented products which inhibits the growth of some bacteria, food pathogens such as *Escherichia coli* and *Listeria monocytogenes* have been reported to survive and grow in fermented milks (Feresu & Nyathi, 1990; Farber & Peterkin, 1991). The presence of food pathogens in fermented milk is usually associated with poor hygienic standards during preparation (Kurmann, 1994). One possible way to limit the growth of food pathogens in fermented milk is the use of protective starter cultures, with the aim of achieving biological preservation without changing the sensory characteristics of the product. Biological preservation refers to extended shelf life and enhanced safety of foods using microorganisms or their metabolites (Lund *et al.*, 2000).

Lactic acid bacteria (LAB) isolated from dairy products has received increased attention as a potential food preservative due to their antagonistic activity against many foodborne pathogens (Kurmann, 1994). The preservative effect exerted by LAB is mainly due to the production of antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins, which can inhibit pathogenic and spoilage microorganisms, extending the shelf life and enhancing the safety of food products (Tamine & Robinson, 1988; Kurmann, 1994; Hutkins, 2006). Organic acids like lactic acid and acetic acid and the presence of hydrogen peroxide reduces intracellular pH (Theron & Lues, 2012). The reduced pH results in unfavourable growth conditions for a wide variety of pathogens and spoilage microbes (Lahtinen *et al*., 2012). The levels and proportions of organic acids produced during fermentation depend on the types of microorganism involved, chemical composition of the culture and the physical conditions encountered during fermentation (Lahtinen *et al*., 2012).

Carbon dioxide creates an anaerobic environment in the fermented milk product which favours the growth of anaerobic LAB and some yeasts, but inhibits obligated aerobic

microorganisms such as mycelial fungi and Gram-negative bacteria (Lindgren & Dobrogosz, 1990; Adams & Nicolaides, 1997; Caplice & Fitzgerald, 1999). A rise in the carbon dioxide pressure may also lead to inefficient cell membrane transport mechanisms, which mediate pH changes of intracellular and extracellular environments and inhibit enzymatic reactions (Caplice & Fitzgerald, 1999).

Bacteriocins are ribosomally synthesized anti-microbial compounds that are produced by many different bacterial species including LAB. Bacteriocins generally exert their anti-microbial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in the death of target cells (Wood & Holzapfel, 1995; Widyastuti & Febrisiantosa, 2014). Nisin is the best-characterised bacteriocin and is used as a food preservative in dairy products, brewing, packaged and canned meats and in sausages world-wide.

LAB strains have been isolated from traditionally fermented Omaere. Omaere is a Namibian traditional fermented dairy product obtained by spontaneous fermentation of unheated bovine milk in a calabash (*Lagenaria siceraria*). Omaere is white, has a low-viscosity with an acidic taste and is consumed as a weaning food, a refreshing drink or with other food products such as porridge. The aim of this study is to determine if the pure LAB cultures isolated from Omaere has any significant antimicrobial effect on the Gram-positive bacteria, *L. monocytogenes* and Gram-negative bacteria, *E. coli* during fermentation.

Materials and Methods

Starter cultures and food pathogens

Pure cultures of *L. plantarum, L. paraplantarum, L. kefiri* and *L. paracasei* subsp. *paracasei* were used as single strain starters. These strains were previously isolated from Namibian traditionally fermented milk Omaere and were frozen with 50% glycerol. In order to revive the strains they were streaked on deMan, Rogosa and Sharpe (MRS) agar (Merck) and incubated for 24 h at 37 °C. Each strain was then picked with a loop from the agar plates and inoculated into 10 ml of MRS broth (Merck) followed by incubation for 24 h at 37 °C to an optical density between 0.08 - 0.1 at a wavelength of 600 nm using a NanoDrop spectrophotometer (Thermo Scientific). These cultures were used as single strain starter cultures to ferment double pasteurised fresh milk at 1% (v/v).

Listeria monocytogenes culture previously isolated from guacamole and *E. coli* ATCC 11775 were used in this study. The bacterial strains were cultured on Listeria Selective Agar (Oxoid) for *L. monocytogenes* and Brilliance E. coli/coliform Selective Agar (Oxoid) for *E. coli*, respectively. Both strains were then picked from the agar plates with a loop and inoculated into 10 ml of Brain Heart Infusion broth (Oxoid) at 37 °C to an optical density of between 0.08 - 0.1. The optical density of the suspension was measured at a wavelength of 600 nm using a NanoDrop spectrophotometer (Thermo scientific). These cultures were inoculated into 50 ml double pasteurised fresh milk to a final concentration of approximately 5 log cfu.ml^{-1}.

Interaction of pathogenic bacteria and *Lactobacillus* spp. during fermentation

To assess the interaction between *L. monocytogenes* and *E. coli* and individual strains of *Lactobacillus* spp. during milk fermentation, single strain pure cultures of *Lactobacillus* spp. were used to ferment milk. One litre of pasteurised fresh milk purchased from a local supermarket in Namibia was placed into sterile glass bottles and pasteurised for 15 min at 80 °C. Fifty ml of the double pasteurised fresh milk was placed in sterile screw capped bottles and inoculated with 1 % (v/v) of each of the four *Lactobacillus* cultures per bottle. *Listeria monocytogenes* or *E. coli* was added to the inoculated milk samples to a final concentration of approximately 5 log cfu.ml^{-1}. Double pasteurised fresh milk with either *L. monocytogenes* or *E. coli* with no starter culture was included as controls. After a gentle agitation, the cultured milks and controls were left to ferment at 37 °C for a maximum of 72 h. Enumeration of viable *L. monocytogenes* and *E. coli* was performed by aseptically pipetting 2 ml of the fermenting milk over time (24 h, 36 h, 48 h and 72 h). One ml of the sample was used to prepare a dilution series of 10^{-1} to 10^{-6} in sterile saline solution (0.85% (w/v) NaCl) in duplicate. One ml of each of these serial dilutions was pipetted in duplicate into appropriately marked petri dishes. Listeria Selective Agar (Oxoid) for *L. monocytogenes* and Brilliance E. coli/coliform selective agar (Oxoid) for *E. coli* was pour plated into these petri dishes and properly mixed for 30 s. All plates were then incubated aerobically at 37 °C for 2 d. The viable microbial counts of *L. monocytogenes* and *E. coli* suspended in each serial dilution were determined and expressed in colony forming units per millilitre (cfu.ml^{-1}) of fermented milk. The change in pH of the fermenting milks was monitored with a pH meter (Mettle Toledo) at 25 °C over time (24 h, 36 h, 48 h and 72 h).

Statistical analysis

Statistical analysis was done using Microsoft excel, 2010. Due to the abnormal nature of the data all enumeration values analysed were transformed to log10. A one way ANOVA was performed on enumeration values (duplicate means) obtained for each sample per medium. If the P (P1) value obtained was significant (P1 value < 0.05) it indicated that at least one of the samples enumeration values differed from another per medium. In that case a two way ANOVA was performed to determine which samples differed significantly (P2<0.05) from each other.

Results and discussions

Listeria monocytogenes

The growth of *L. monocytogenes* was influenced by the LAB starter cultures over time (Fig.10). After 24 h of fermentation an increase in the growth of *L. monocytogenes* (1 - 4 log cfu.ml^{-1}) was observed in both the milk with the starter cultures and the control that did not contain starter culture. According to Delgado *et al. (*2001) the inhibitory action of LAB is due to the accumulation of main primary metabolites (lactic and acetic acids, ethanol and carbon dioxide), as well as to the production of other antimicrobial compounds, such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins. Therefore, this result suggests that not enough metabolites and antimicrobial compounds from the chosen LAB were accumulated in the milk after 24 h of fermentation to have any lethal effect on *L. monocytogenes*. However, after 36 h of fermentation the *L. monocytogenes* concentration was reduced by 2 log cfu.ml^{-1} and after 48 h of fermentation none was detected in the milk with *L. plantarum, L. paraplantarum* and *L. paracasei* subsp. *paracasei* used as single strain starters. The milk fermented with *L. kefiri* after 36 h of fermentation showed the concentration was reduced by 2 log cfu.ml^{-1}, but after 48 h of fermentation the concentration was reduced by 4 log cfu.ml^{-1} and was only undetected after 72 h. In the control the concentration of *L. monocytogenes* was only reduced by 2.8 log cfu.ml^{-1} after 72 h of fermentation. This result suggests that the LAB strains used in this study had a pronounced inhibitory effect on the *L. monocytogenes* strain used in this study during milk fermentation. The one way ANOVA performed on all the enumeration values on Listeria Selective Agar gave a P-value of 0.01, indicating that at least one of the fermented milk sample enumeration values differed significantly from another. Therefore the one way ANOVA was followed by a two way ANOVA

to determine which fermented milk sample enumeration values on Listeria selective agar differed significantly from another. Control enumeration values differed significantly from all the enumeration values obtained from the milk samples fermented in the presence of LAB on Listeria Selective Agar (Table 6). This can be due to the fact that *L. monocytogenes* reached undetectable levels in all the milk fermented in the presence of LAB at the end of fermentation (72 h) but it was still detected in the control. Since the enumeration values obtained from milk fermented with *L. plantarum* and *L.kefiri* on Listeria Selective Agar differed significantly from each other, it suggests that the eradication of *L. monocytogenes* does not only depend on the fermentation time but also on the LAB strains predominant in the starter culture.

The results obtained in this study are similar to the results obtained in the study by Ashenafi. (1994). It was found *L. monocytogenes* strains increased slightly in concentration from 1.0 to 1.7 log units for the first 12 h of fermentation of Ergo. After 12 h of fermentation, the counts decreased steadily to undetectable levels at 48 h or 60 h.

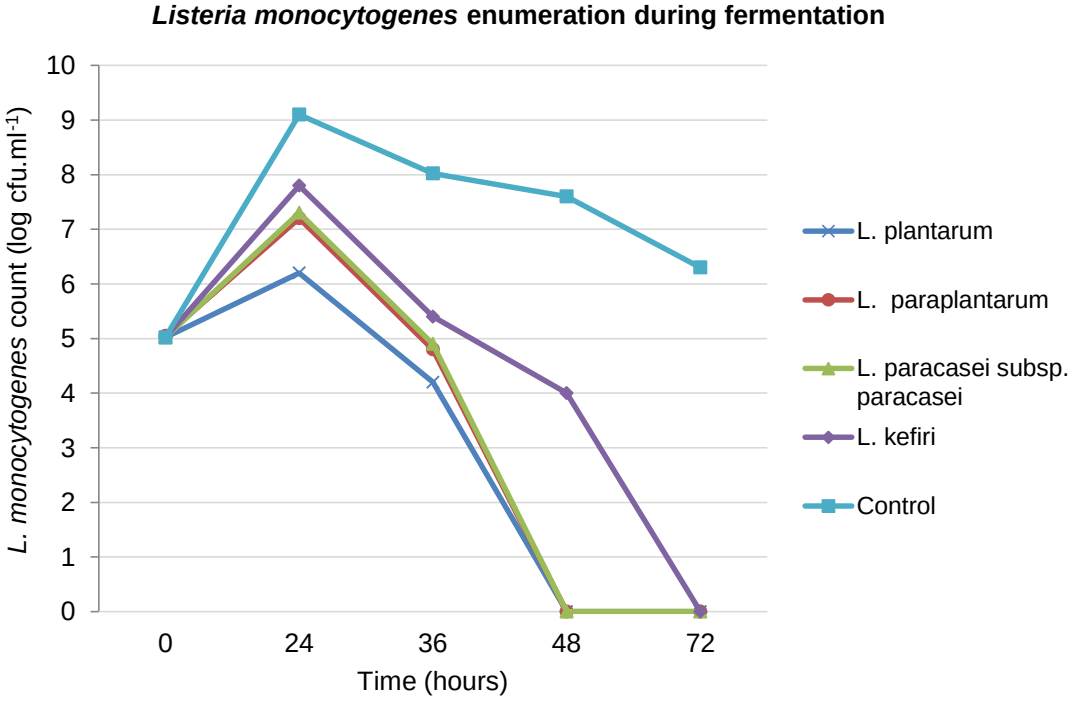

Figure10 *Listeria monocytogenes* enumeration during milk fermentation in the presence of different LAB starter cultures (*L. plantarum*, *L. paraplantarum, L. casei* subsp*. paracasei* and *L. kefiri*) and the control in the absence of starter culture.

Table 6 P2-values for *L. monocytogenes* counts over time in the presence of each strain during fermentation

	L. plantarum	***L. paraplantarum***	***L. paracasei* subsp. *paracasei***	***L. kefiri***	**Control**
L. plantarum		0.053[b]	0.06[b]	0.02[a]	0.00[a]
L. paraplantarum	0.053[b]		0.11[b]	0.07[b]	0.00[a]
L. paracasei subsp. *paracasei*	0.06[b]	0.11[b]		0.08[b]	0.00[a]
L. kefiri	0.02[a]	0.07[b]	0.08[b]		0.00[a]
Control	0.00[a]	0.00[a]	0.00[a]	0.00[a]	

[a] P2 value < 0.05 indicates that at least one of the mean values differs significantly from another using two way ANOVA test.

[b] P2 value >0.05 indicates the mean values does not differ significantly from another using two way ANOVA test

The pH changes during fermentation using *L. plantarum, L. paraplantarum, L. paracasei* subsp. *paracasei* and *L. kefiri* in the presence of *L. monocytogenes* were very similar. A decrease in pH value was observed from 6.75 to 4.69, 4.24, 4.41 and 4.91, respectively after 24 h. After 72 h, when no *L. monocytogenes* was detected the pH of the milk was 3.82, 3.69, 3.71 and 3.97, respectively (Fig. 11). A different pH profile was observed with the control. After 24 h of fermentation the pH was reduced slightly from 6.75 to 6.23 and at the end of the fermentation at 72 h the pH of this milk was 4.16. This result suggests that fast acid production by the studied LAB played a significant role in the elimination of *L. monocytogenes* during fermentation.

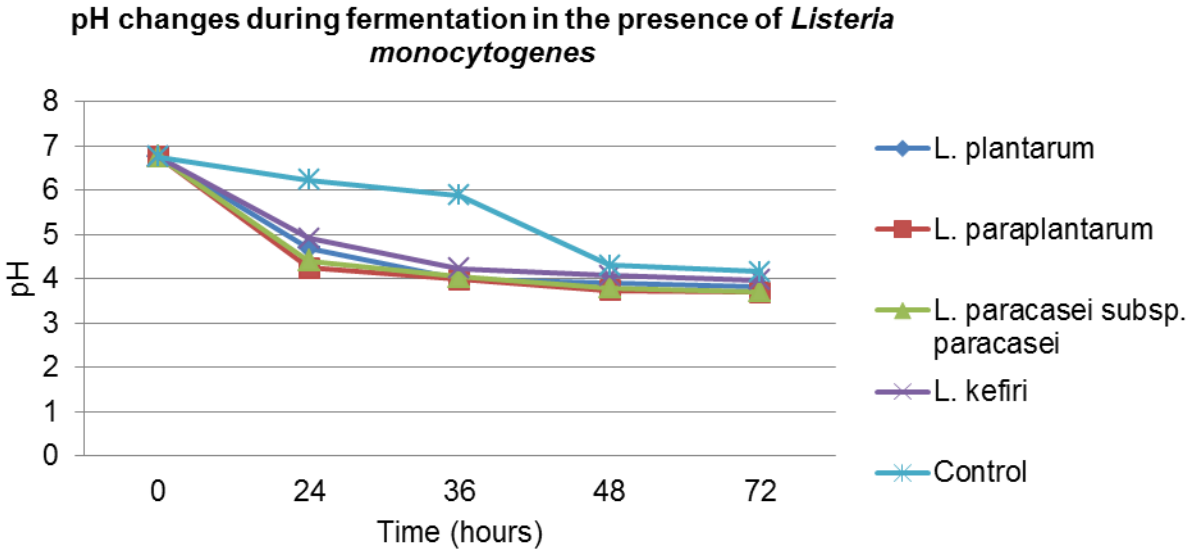

Figure 11 Changes in pH during milk fermentation in the presence of *L. monocytogenes* and different LAB starter cultures (*L. plantarum, L. paraplantarum, L. parcasaei* subsp. *paracasei* and *L. kefiri*) and the control in the absence of starter culture.

Escherichia coli

The growth of *E. coli* was influenced by the starter cultures over time (Fig. 12). After 24 h of fermentation an increase in the growth of *E. coli* (2 - 3 log cfu.ml^{-1}) was observed in both the milk with the starter cultures and the control with no starter culture. This suggests that the time of fermentation is an important factor in the antimicrobial activity of LAB during fermentation. Similar trends were observed in the milk with *L. plantarum, L. paraplantarum* and *L. paracasei* subsp. *paracasei* after 48 h of fermentation as the *E. coli* concentration was reduced by 4 log cfu.ml^{-1} and after 72 h of fermentation no *E. coli* was detected. These results suggest that the LAB strains had inhibitory effect on the *E. coli* strain used in this study during milk fermentation and this inhibitory effect depended on the fermentation time. The one way ANOVA performed on all fermented milk samples gave a P-value of 0.00 indicating that at least one of the fermented milk samples differed significantly from another. Therefore, the one way ANOVA was followed by a two way ANOVA to determine which fermented milk sample differed significantly from another. There were no significant differences observed between the count of *E. coli* over time in the milk fermented with *L. plantarum, L. paraplantarum* and *L. paracasei* subsp. *paracasei* but differed significantly from the milk fermented with *L. kefiri* and the control (Table

7). In the milk fermented with *L. kefiri* after 48 h the concentration of *E. coli* was decreased by only 1 log cfu.ml^{-1} and at the end of the 72 h the concentration was only reduced by 1.7 log cfu.ml^{-1}. In milk fermented without the addition of starter the concentration of *E. coli* was only reduced by 1.6 log cfu.ml^{-1} after 72 h of fermentation. From this result it can be concluded that the strain of *E. coli* is not sensitive to the antimicrobial components produced by *L. kefiri* in comparison with the rest of the LAB used in this study.

The inactivation of *E. coli* when introduced into fermented products was observed by Mufandaedza *et al*. (2006) who reported 2 to 3 log cfu.ml^{-1} reduction of *E. coli* 3339 after 48 h when inoculated into fermented milk using single and paired starter strain combination obtained from Zimbabwean traditional fermented milk. Ogwaro *et al*. (2002) indicated that *E. coli* 0157:H7 was inactivated after 96 h in traditional African yoghurt at ambient storage, whereas inactivation took about 144 h at refrigeration temperature. After 5 days of fermentation *E. coli* was reduced by 2.7 log cfu.ml^{-1} during the production of madila, traditional milk in Botswana (Parry-Hanson *et al*., 2009). The sensitivity of *E. coli* to milk fermentation seems to vary according to time and temperature of fermentation, LAB present and the strain of *E. coli* used.

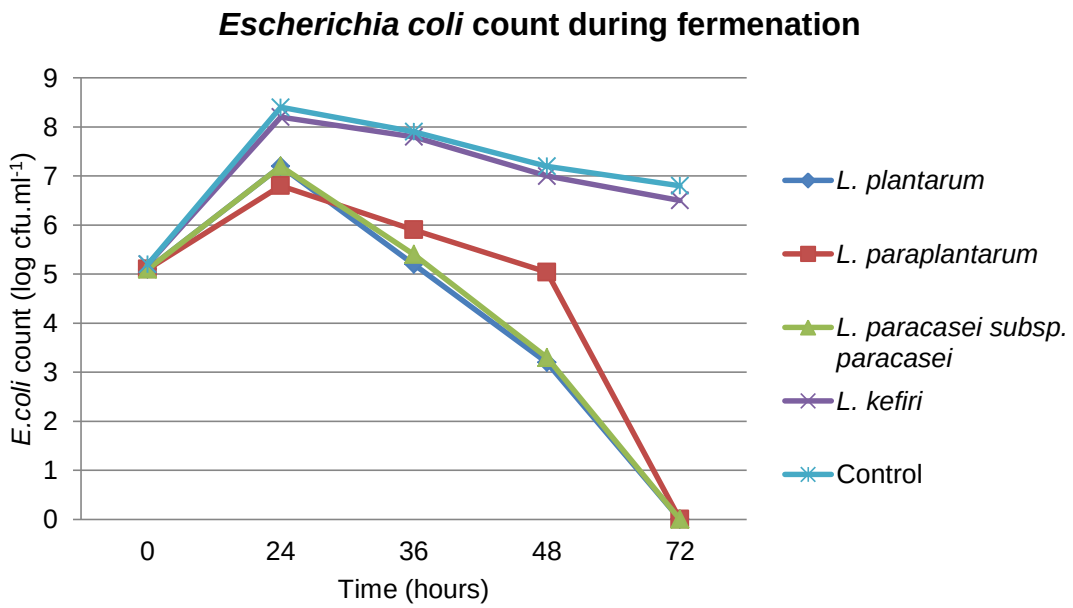

Figure 12 *Escherichia coli* concentration during milk fermentation in the presence of different LAB starter cultures (*L. plantarum*, *L. paraplantarum*, *L. casei* subsp. *paracasei* and *L. kefiri*) and the control in the absence of starter culture.

Table 7 P2-values for *E. coli* enumeration over time in the presence of each strain during fermentation

	L. plantarum	***L. paraplantarum***	***L. paracasei* subsp. *paracasei***	***L. kefiri***	**Control**
L. plantarum		0.12[b]	0.09[b]	0.01[a]	0.00[a]
L. paraplantarum	0.12[b]		1.00[b]	0.01[a]	0.01[a]
L. paracasei subsp. *paracasei*	0.09[b]	1.00[b]		0.00[a]	0.00[a]
L. kefiri	0.00[a]	0.01[a]	0.00[a]		0.01[a]
Control	0.00[a]	0.01[a]	0.00[a]	0.01[a]	

[a] P2 value < 0.05 indicates that at least one of the mean values differs significantly from another using two way ANOVA test.

[b] P2 value >0.05 indicates the mean values does not differs significantly from another using two way ANOVA test

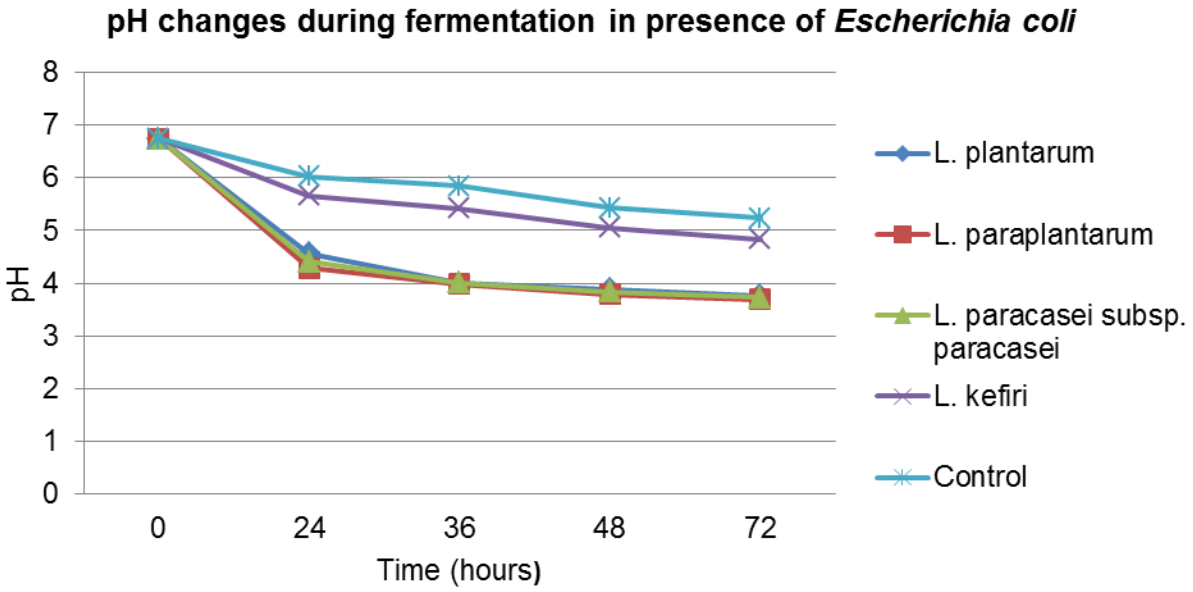

Figure 13 Changes in pH during milk fermentation in the presence of *E.coli* and different lactic acid bacteria starter cultures (*L. plantarum, L. paraplantarum, L. parcasaei* subsp. *paracasei* and *L. kefiri*) and the control in the absence of starter culture.

The pH changes during fermentation using *L. plantarum, L. paraplantarum* and *L. paracasei* subsp. *paracasei* in the presence of *E. coli* were similar and were reduced from 6.75 to 4.57, 4.29, and 4.41, respectively after 24 h (Fig. 13). After 72 h, the pH of this milk was 3.76, 3.69 and 3.73, respectively. A different pH profile was observed with the milk fermented with *L. kefiri*, after 24 h of fermentation the pH was reduced slightly from 6.75 to 5.66 and at the end of the fermentation at 72 h the pH was 4.83. This suggests that fast acid production by *L. plantarum, L. paraplantarum* and *L. paracasei* subsp. *paracasei* played a significant role in the elimination of *E. coli* during fermentation.

Conclusion

Three of the four LAB strains used in this study namely, *L. plantarum, L. paraplantarum, L. parcasaei* subsp. *paracasei* inhibited the growth of Gram-positive bacteria, *L. monocytogenes* and Gram-negative bacteria, *E. coli* during milk fermentation. The inhibition activity may have been due to fast acid production by the studied LAB and possibly the production of bacteriocins. These LAB strains are possible candidates for the formulation of bio-protective starter cultures that can be employed for production of safe and potentially probiotic fermented milk products with unique tastes, aromas and characteristics. Further studies will be necessary to elucidate the types of antibacterial agents that are produced by these LAB strains and the optimal conditions for antagonistic activity should be evaluated.

The *L. kefiri* strain used in this study took longer than the other LAB strains to inhibit the growth of the Gram-positive bacteria, *L. monocytogenes* and failed to inhibit the growth Gram-negative bacteria, *E. coli* and, therefore, is not recommended to be used as a single strain starter but may be used in combination with other LAB strains.

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Chapter 5
General Discussion and Conclusions

Escherichia coli and *Listeria monocytogenes* are considered the most common foodborne pathogens that are present in milk. Their presence can lead to foodborne diseases through consumption of contaminated milk and they are able to survive in fermented milk products (Krause & Hendrick, 2011). A possible approach to naturally improve food safety, quality and shelf-life of foods is to search for those lactic acid bacteria (LAB) that would be able to suppress the growth of food pathogens and, at the same time, would be inherent to food products (Kurmann, 1994). The aim of this study was to isolate and identify LAB and yeasts from Namibian traditionally fermented milk Omaere and to study the effect of selected identified LAB on *L. monocytogenes* and *E. coli* during fermentation.

The microbial consortium present in five samples of Namibian traditionally fermented milk Omaere was isolated and enumerated on six different selective media that included deMan, Rogosa and Sharpe Medium (MRS) supplemented with cycloheximide for lactobacilli (MRS+C), MRS supplemented with vancomycin for leuconostocs (MRS+V), MRS supplemented with ethanol for acetic acid bacteria (MRS+E), M17 agar for lactococci, and Chloramphenicol Glucose Agar (CGA) and Potato Dextrose Agar (PDA) for yeasts. The highest enumeration values obtained from Omaere samples 2 and 3 were from MRS+V used for the growth of *Leuconostoc* spp. However, for Omaere samples 1, 4 and 5 the highest values were obtained from MRS+C used for the growth of Lactobacilli. The Harrison Disc method was used to select bacterial and yeasts colonies for further testing. The selected microbes were identified by using PCR amplification followed by DNA sequencing. The only LAB found in Namibian fermented milk Omaere belonged to the genus *Lactobacillus,* with the predominant species *Lactobacillus plantarum* (52%) and in lesser numbers *Lactobacillus paracasei* subsp. *paracasei* (12%), *Lactobacillus paraplantarum* (8%), *Lactobacillus kefiri* (8%) and *Lactobacillus casei* (2%). The yeasts isolated were *Kazachstonia unispora* formerly known as *Sacchromyces unisporus* (9%), *Saccharomyces cerevisiae* (8%) and *Candida pararugosa* (2%).

Single strain pure cultures of the *Lactobacillus* spp. isolated were used to ferment milk inoculated with *L. monocytogenes* and *E. coli* and their interaction was monitored over time. After 48 h of fermentation no *L. monocytogenes* was detected in the milk inoculated with the selected starter cultures, with the exception of *L. kefiri* where undetected levels of the pathogen was only reached after 72 h of fermentation. A different profile was observed with the milk inoculated with *E. coli*. These bacteria were not detected in milk inoculated with the starter

culture only after 72 h of fermentation. In the presence of *L. kefiri* after 72 h of fermentation *E. coli* concentrations was only reduced by 1.7 log. In milk fermented without the addition of starters the concentration of *L. monocytogenes* was only reduced by 2.8 log after 72 h of fermentation. This work has shown that the microorganisms involved in the production of traditionally fermented Omaere comprises a combination of *Lactobacillus* spp. and yeasts. Three of the *Lactobacillus* spp. namely *L. plantarum, L. paraplantarum, L. parcasaei* subsp. *paracasei had* antimicrobial effects against *L. monocytogenes* and *E. coli* and, therefore , has the potential to be used as starter cultures in the production of commercial microbiologically safe fermented milk products. *L. kefiri* strain used in this study took longer to eliminate or failed to eliminate the food pathogens used in the study and, therefore, not recommended to be used as starter culture.

Concluding remarks

Identification of microbial isolates after enumeration was important before the microbial consortium present in the Omaere samples could be described as the selective media used were not highly selective for specific microbes. The data obtained in this study shows that three of the four *Lactobacillus* spp. isolated from the Namibian traditionally fermented milk Omaere had antimicrobial effect against the foodborne pathogens used in this study. This antimicrobial effect was associated with fast acid production by this LAB and dependent on the type of LAB present and time of fermentation. After further characterization of the antimicrobial activity these cultures can be used to develop bio protective starter cultures. As a result microbiologically safe, new and original fermented dairy products with unique tastes, aromas and characteristics can be produced on an industrial scale.

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